RELATIONSHIPS OF AMPHOLYTES TO ASSIMILATION AND RECOVERY OF AMMONIUM AND NITRATE NITROGEN IN PLANT TISSUE

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Introduction

In experiments on nitrate and ammonium assimilation (**20, 21, 22**), the determination of the respective ions in plant tissue resulted in values which indicated that they were not being satisfactorily recovered by the accepted methods (**18**). This was particularly true for plants grown wholly or in part with ammonium nitrogen as the nitrogen source.

In a previous paper (**20**) the term "combined ammonia" was used to designate a nitrogenous fraction which was recovered by aspiration with sodium hydroxide. This was an ammonium ion, which was slowly liberated after the so-called free ammonium ions had been removed with a weak alkali (sodium carbonate). In tomato plants grown with nitrate nitrogen this fraction was usually negligible, but in those grown with ammonium nitrogen (**22**) it was particularly large. No explanation has been given for this phenomenon. The possibility that the "combined ammonia" originated as a disintegration product of glutamines is not borne out by the data. They show that, in addition to "combined ammonia," there was present also a "combined nitrate" fraction which could only be recovered by taking cognizance of LOEB's theory (**5**) of ion displacement in a colloidal system; that is, the ammonium and nitrate ions apparently formed weakly dissociated salts with soluble and insoluble organic nitrogenous compounds (ampholytes) which were present in the aqueous extract of tomato-plant tissue as well as in the protein-free extract. That this is possible has been shown by ROBBINS (**17**). The hydrogen-ion concentration of the nutrient solution supplied to plants in sand culture and differences in pH of the extracting water in the tissue analysis caused variations in the amounts of both nitrate and ammonium ions that were recovered.

When an aliquot of extract, from plants grown with ammonium nitrogen and from which the coagulable protein had been removed, was aspirated for ammonium or nitrate nitrogen, the ammonium ion was extracted only after 12 or more hours of aspiration instead of the 2- to 3-hour period as recommended (**18**). When a known quantity of a pure ammonium salt was aspirated with a mild alkali (sodium carbonate), the ammonium ion was completely recovered in 2–3 hours. However, the aspiration of aliquots of protein-free extract from plants grown with ammonium nitrogen required 30 or more hours before the last traces of ammonium nitrogen were displaced. Tomato plants grown with nitrate nitrogen usually have so little ammonium present (**20**) that the slow recovery observed above is easily overlooked. There is thus considerable evidence that amino acids (glycine found in plants by PEARSALL and EWING [**14**]) and proteins in protoplasm could react with ions absorbed from a nutrient solution.

That this occurs in plant tissue by virtue of the amphoteric nature of constituents in the protoplasm was suggested by ROBBINS (17).

Investigation

Data are presented which show the amphoteric properties of amino acids, proteins, and other plant materials (table 1). Whether nitrate or ammonium ions from ammonium nitrate combine with these materials depends on the pH of the solution in which they are dispersed or dissolved, as ROBBINS (17) has pointed out. Above pH 4.0, asparagine combined with ammonium ions, liberating nitrate ions and causing equilibrium in the solution to be established at a lower pH. In a solution more acid than pH 4.0, asparagine combined with the nitrate ions, liberating ammonium ions, with equilibrium established at a less acid pH. Glycine has an isoelectric point at pH 6.1 and reacts in a similar manner to asparagine above and below this point. Data from pure casein (fig. 1) also show how an amphoteric substance responds at different pH values. Thus it is probable that many substances found in protoplasm, and with widely different isoelectric points, have these amphoteric properties.

If ammonium and nitrate ions form dissociation compounds with ampholytes in the cell, theoretically it should be possible to displace different amounts of the ions from plant tissue by extracting it with water at different pH values. That this is possible is shown by the data in figure 2. They were obtained by placing

TABLE 1

CHANGES IN PH AFTER ADDITION OF AMPHOLYTES TO 50-ML. STOCK SOLUTIONS OF AMMONIUM NITRATE PREVIOUSLY ADJUSTED TO INDICATED PH VALUES WITH NITRIC ACID OR AMMONIUM HYDROXIDE

Initial pH of stock solution	Kind and amount of ampholyte added					
	Asparagine, 0.5 gm.	Glycine, 0.5 gm.	Casein isoelectin, 3.5 gm.*	Pectin commercial, 0.5 gm.	Calcium pectate, 0.5 gm.	Soybean protein, 2.0 gm.
1.9	.....	+2.1	+1.5	+1.4	+1.3	+1.9
2.2	+1.2	.....	+1.9	+1.1	+1.6	+2.2
2.8	.....	+1.2	+1.8	+0.7	+1.9	+2.2
3.0	.....	+1.3	+1.7	+0.6	+2.2	+2.2
3.2	.....	+1.2	+1.6	+0.4	+2.8	+2.2
3.5	+0.5	.....	+1.4	0.0	+3.0	+1.9
4.0	0.0	+1.1	+0.8	−0.4	+2.8	+1.6
5.6	−1.6	+0.2	−0.6	−2.1	+1.2	0.0
6.8	.....	−0.5	−1.6	−3.6	+0.3	−1.2
7.6	−3.3	.....	−2.2	−4.1	−0.1	−1.6
8.3	.....	−1.1	−2.3	−4.7	−0.4	−2.1
8.9	−2.5	−1.3	−2.8	−5.1	−0.3	−2.7
9.6	.....	−1.4	−3.1	−5.8	−0.2	−2.8
10.2	−2.4	.....	−3.0	−5.4	−0.1	−2.0
10.3	.....	−1.6	−2.4	.....	.....	−0.2
Exchange neutrality (pH)	4.0	6.1	4.7	3.5	7.4	5.6

* Stock solution of casein isolectin = 100 ml.

100-gm. aliquots of ground tomato tissue, from plants grown in soil, in 800 ml. of distilled water and adjusting with HCl or NaOH to the indicated pH values. After 30 minutes the liquid was pressed out as much as possible, the residue being washed twice with 100 ml. of water. Soluble proteins were not coagulated. The

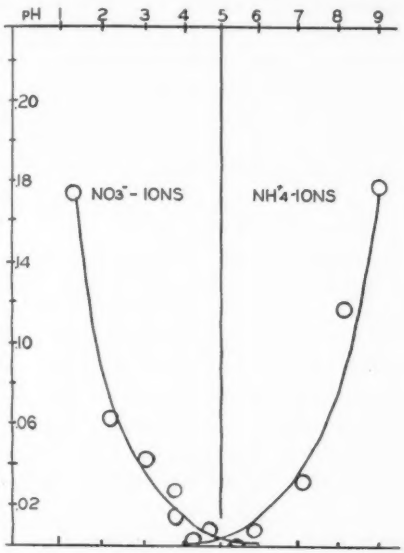

FIG. 1.—Absorption of nitrate and ammonium ions by casein in solutions of ammonium nitrate adjusted to different pH values. Figures on left are gm. of ions in 100 gm. dry matter absorbed by casein. Intersection of curves is at isoelectric point of casein.

data show that, as the pH is lowered to 3.0, less and less nitrate is extracted (less is dissociated from ampholytes). Much larger quantities were extracted at pH 10. On the other hand, less ammonium nitrogen was recovered as the pH value was increased. In other words, with change in pH either the ammonium or the nitrate ions apparently become dissociated from the amphoteric substances in the tissue so that there is no one pH value at which all the nitrate or ammonium ions will be easily recovered. In this particular experiment, when tissue was extracted in distilled water at pH 5.4 (unadjusted), less than half the nitrate but at least 75% of the ammonium nitrogen was recovered. That the remaining nitrate and ammonium nitrogen was combined with the coagulable protein or amino acids is shown by a comparison of two methods of extraction—namely, aqueous extraction and electrodialysis—of young green tomato stems which had been ground and divided into three 100-gm. aliquots. The data (table 2) show

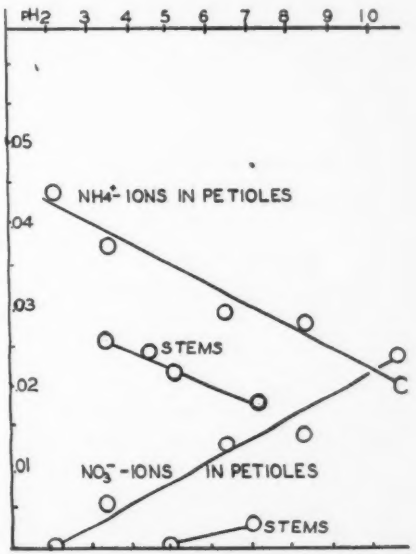

FIG. 2.—Nitrate and ammonium ions extracted from young tomato plant tissue in 800 ml. of water for 30 minutes at indicated pH values. Figures on left represent gm. of nitrate or ammonium nitrogen removed from 100 gm. of green tissue.

that more nitrate and ammonium ions were removed by electrodialysis than by aqueous extraction and, furthermore, that the coagulated protein removed from the aqueous extract contained some nitrate but no ammonium nitrogen.

Three groups of tomato plants were grown in soil adjusted to pH 4.0, 6.0, and

7.4. Each group of plants was divided into three lots which were supplied, respectively, with nutrient solutions containing ammonium sulfate, sodium nitrate, or ammonium hydroxide. Samples of leaf tissue were removed when the plants were 10–12 inches tall and were analyzed by electrodialysis as well as by aqueous extraction. For plants supplied with nitrate nitrogen there was very little difference in the amounts of ions recovered by the two methods of extraction (fig. 3). When grown with ammonium sulfate, the method of extraction affected the recovery of ammonium nitrogen from plants grown at pH 4.0 but not from those grown at pH 6.0 and 7.4. This may be explained by the differences in growth and in the rate of nitrogen assimilation in plants rooted in soils of different pH values (22). The nitrate nitrogen recovered by the two methods of extraction showed large differences in plants grown with either ammonium sulfate or ammonium hydroxide.

TABLE 2

NITRATE AND AMMONIUM IONS EXTRACTED FROM TOMATO-STEM TISSUE BY VARIOUS METHODS

SAMPLE	METHOD OF EXTRACTION	MGM. IN 100 GM. GREEN TISSUE	
		Nitrate	Ammonium
1	Electrodialyzed	510	49
2a	Aqueous extraction	347	27
2b	Coagulated proteins from 2a	14	0
3	Aqueous extraction		
3a	Electrodialyzed before removal of proteins	497	51
3b	Electrodialyzed after removal of proteins	354	34

Tomato plants were grown in sand cultures at different pH values maintained by continuously flowing nutrient solution. The data shown in figure 4 were obtained from plants supplied with calcium nitrate as the source of nitrogen. The recovery of ammonium nitrogen showed little difference between the two methods of extraction, but the method

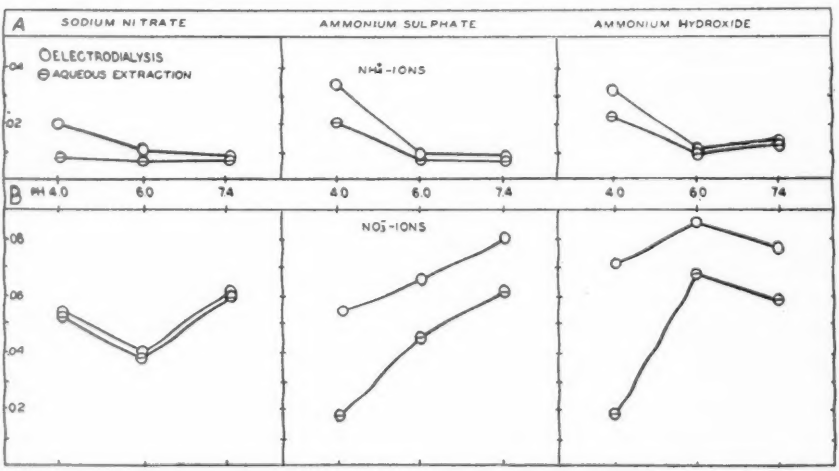

FIG. 3.—Nitrate and ammonium ions recovered from tomato-plant tissue by two methods of extraction from plants grown on soils having three different pH values. Figures on left represent gm. of nitrate or ammonium ions in extracts from 100 gm. of green tissue.

used had considerable influence on the amount of nitrate nitrogen recovered from plants grown at pH 3.5, 4.5, and 7.5. Apparently nitrate ions were combined with ampholytes in the plants grown at pH 3.5, 4.5, and 7.5. It is interesting to note that the point of exchange neutrality[1] for tomato-stem tissue is between pH 5.5 and 6.5. It would seem that nitrate ions can be easily recovered from such tissue by aqueous extraction between these points.

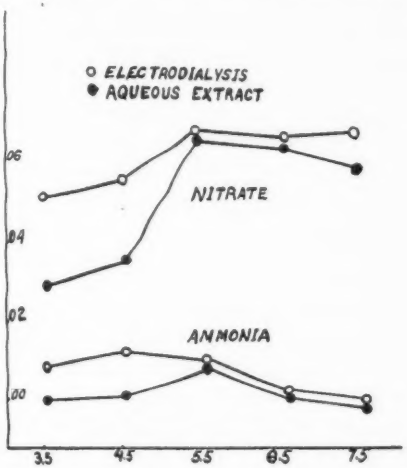

FIG. 4.—Amounts of nitrate and ammonium nitrogen recovered by two methods of extraction of tomato plant tissue. Plants grown in sand with calcium nitrate as source of nitrogen. Scale represents gm. in 100 gm. of green tissue.

In figure 5 are shown similar data for roots, stems, petioles, and leaf blades of tomato plants grown in sand culture with nitrate nitrogen. Extraction of samples representing the four regions was carried out under uniform conditions; no attempt was made to change the natural pH of each extract. More nitrate nitrogen was extracted in each case by electrodialysis than by aqueous extraction; the differences were 119% in the roots, 126% in the stems, 43% in the petioles, and 126% in the leaf blades. The total amount of nitrate varied; it was highest in the roots and least in the blades. The percentage amount of combined nitrate is correlated with the comparative amount of protein nitrogen in the roots, stems, and leaves of tomatoes grown with sodium nitrate, as shown graphically in figure 2 of an earlier publication (22). In leaves, with five times as much protein nitrogen as in roots, there was 126% more nitrate recovered by electrodialysis than by aqueous extraction. The roots and stems had approximately the same percentage content of protein, but the percentage increase in nitrate nitrogen extracted by electro-

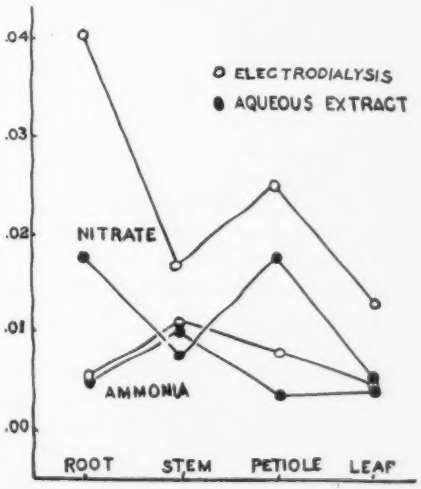

FIG. 5.—Amounts of nitrate and ammonium nitrogen removed by electrodialysis and aqueous extraction from different tissues of tomato plants grown in sand culture with no attempt to control pH of nutrient solution. Scale represents gm. in 100 gm. of green tissue.

[1] The point of exchange neutrality is used to designate that pH value at which the free ampholyte combines with an equal number of cations and anions in a particular salt solution in exchange for hydrogen and hydroxyl ions.

dialysis over aqueous extraction was slightly higher in the stems than in the roots. There is considerable difference between fine-root and stem tissues in the types of proteins and amino acids found.

Discussion[2]

MICHAELIS (8) has shown that casein and gelatin have isoelectric points at approximately pH 4.7 and that these proteins forms dissociable compounds with anions in a solution having a pH value above the isoelectric point. MATTSON (7) carried this work further and found that the laws governing the soil ampholytoids —that is, the chemical behavior of the "silicated, phosphated and humated compounds of the sesquioxides"—may be extended to proteins in the soil which are in effect derived from plants and are part of the organic matter of the soil. Proteins, then, when in the presence of anions and cations at any given pH, will form compounds which dissociate either anions or cations, depending on their isoelectric points.

There is some evidence that other than nitrate and ammonium ions are combined in plant cells. LUTEMAN and WALBRIDGE (6) suggested that magnesium is found in combined and uncombined forms. Whether the combined magnesium is anything more than that present in the chlorophyll molecule is not clear; since metallic ions do associate with organic matter in the soil (7), we may assume that they also do so in protoplasm. NIGHTINGALE *et al.* (9) have shown that calcium may be combined with the protein complex and that, when calcium-deficient plants, which have stopped growing because of a lack of soluble calcium, are placed in continuous darkness, proteolysis releases a sufficient amount of usable calcium so that growth may be resumed. NIGHTINGALE *et al.* (12) suggested the possibility that potassium may be held by protein, although they recovered practically all the potassium by water extraction. This, however, does not prove that potassium may not be so combined. They (13) found no release of sulfate ions when the residue from water extraction was electrodialyzed. Here again the hypothesis that ions combined with ampholytes was not disproved.

In view of the fact that the data show a chemical combination between the ampholytes of the cell and the absorbed common ions or electrolytes (nitrate and ammonium ions), it is of interest to know whether the relationship of this phenomenon to the assimilation of nitrate and ammonium ions is more than a buffer system in the plant and whether it may account for observed phenomena in the determination of ammonium and nitrate. The following observations indicate the existence of such dissociation complexes in tissue extracts: (*a*) Electrodialysis in many cases recovers more anions and cations than is possible by aqueous extraction (figs. 2, 3). (*b*) The quantity of nitrate and ammonium ions recovered by aqueous extraction of tissue depends on the pH of the extracting fluid (fig. 2). (*c*) The recovery of ammonium ions from aqueous extraction by aspiration with weak alkali is a prolonged process compared with recovery from a pure ammonium salt. (*d*) The aspiration method of SESSIONS and SHIVE (18), using sodium carbonate for ammonium and sodium hydroxide for nitrates, recovers additional ammonium ions with sodium hydrox-

[2] In this discussion only nitrate and ammonium ions are used for illustrating certain reactions. Other anions and cations would, of course, be present and would complicate the reactions in the cell. Whether other anions and cations behave in a similar manner requires further investigation. Preliminary indications are that they do.

ide which apparently are not derived from the reduction of nitrate ions.

These observations would indicate that the chemical attraction between ampholytes and the common ions not only is a phenomenon characteristic of the proteins, as suggested by THERON (19) and LOEB (5), but seems associated with the soluble ampholytes as well. A mechanism has been suggested by LOEB (5) which shows how these ions may be combined with protein:

$$R<\begin{matrix}NH_2\\COOH\end{matrix} + HNO_3 \rightleftharpoons R<\begin{matrix}NH_3NO_3\\COOH\end{matrix}$$

$$R<\begin{matrix}NH_2\\COOH\end{matrix} + NH_4OH \rightleftharpoons R<\begin{matrix}NH_2\\COONH_4\end{matrix} + H_2O.$$

MICHAELIS (8) listed a number of amino acids which occur in plants; their isoelectric points range from pH 2.76 for aspartic acid to pH 10.97 for arginine. Some of these are placed on the graph in figure 6, showing the theoretical curves for ammonium and nitrate absorption and accumulation in growth processes.

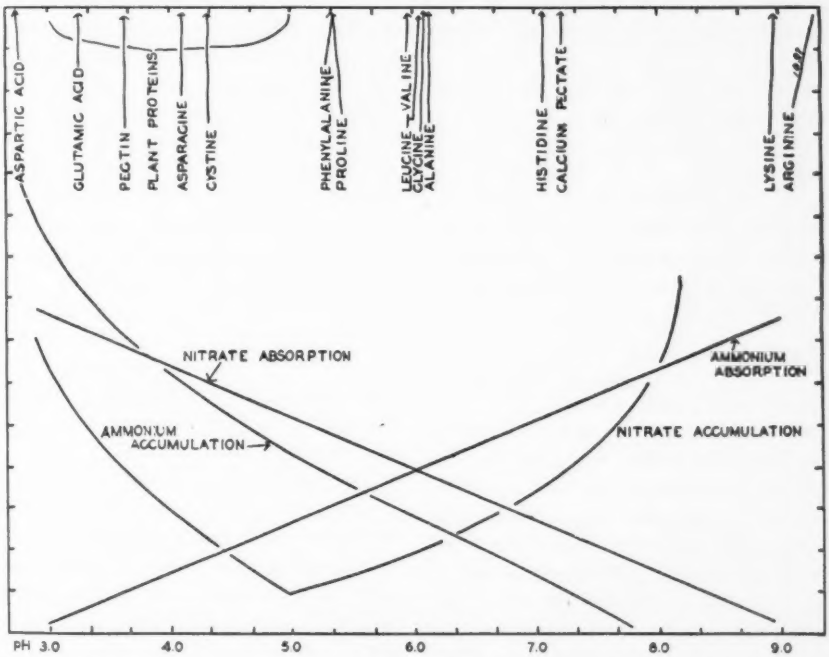

FIG. 6.—Distribution of amino acids on pH scale according to isoelectric points, and theoretical curves showing absorption and accumulation of nitrate and ammonium nitrogen in plant tissue adjusted to different pH levels. It shows possible role that amino acids play in adsorption of nitrate and ammonium nitrogen. At points more acid than their isoelectric points they adsorb nitrates and at points more alkaline they adsorb ammonia. Scale represents gm. per 100 gm. of dry matter.

If aspartic acid (pH 2.76) and asparagine (pH 4.1), isoelectric below pH 4.2, exist as free ampholytes in the cell, they would combine primarily with ammonium ions (or cations in general) at any pH of the sap above their isoelectric points. The hydrogen of the ampholyte would be displaced by the ammonium ion. Arginine (pH 10.97), lysine (pH 9.4), and

histidine (pH 7.15)—amino acids which PEARSALL and EWING (14) reported as being present in the potato—have isoelectric points above 7.0 and would combine primarily with nitrate ions and with anions in general if the pH of the cell sap was more acid than pH 7.0, although histidine may combine with cations in rapidly dividing parenchymatous cells which ECKERSON (2) stated may have a pH of 7.4.

The following reactions may take place: Suppose asparagine and aspartic or glutamic acids, with isoelectric points lower than pH 4.2, occur in tomato sap at pH 5.4. They would dissociate hydrogen ions, whereas dissociation of the hydroxyl ions would be suppressed.[3] They would react with ammonium nitrate as follows:

$$NH_2RCOOH + NH_4NO_3 \rightarrow NH_2RCOONH_4 + HNO_3 .$$

The ammonium ion would form a weakly dissociated salt with the ampholyte. The sap would tend to become more acid because of the free nitric acid. If histidine, lysine, and arginine, with isoelectric points above pH 7.0, also occur in the tomato juice, they would dissociate hydroxyl ions, while dissociation of hydrogen ions would be suppressed. They would react with ammonium nitrate as follows:

$$NH_2RCOOH + NH_4NO_3 \rightarrow NH_3NO_3RCOOH + NH_4OH .$$

The nitrate ions would form weakly dissociated compounds with the ampholytes. The sap would tend to become more alkaline. The free acid and ammonium hydroxide would neutralize each other, and any change in reaction would depend on the amount of ampholytic material having isoelectric points at different pH values.

The process—ampholytic behavior of protoplasm—in the plant cell, however, is not so simple as these reactions would indicate. A large number of amino acids having isoelectric points between pH 5.0 and 6.5—such as phenylalanine, cystine, tyrosine, valine, leucine, alanine, and proline—would dissociate hydrogen, hydroxyl, or protein (ampholyte) ions, depending on the pH of the sap of any particular tissue. These ions would enter into chemical combination with electrolytes and form dissociable compounds. The pH values of the isoelectric points of these amino acids are so close to the pH values of sap in many cells that they might change their charge several times during a day since, according to INGALLS and SHIVE (4), the composite pH of cell sap fluctuates considerably.

The data suggest that three types of nitrate and ammonium ions are present in plants which are absorbing these ions from a nutrient solution: (*a*) free ions in solution as mineral salts; (*b*) ions associated with amino acids and other soluble plant products which may be quite mobile; and (*c*) ions associated with proteins and other materials which are in a more or less colloidal condition or may even be insoluble and become part of the reserve material in the plant. If this assumption is correct, it offers a possible explanation why a DONNAN equilibrium hypothesis (1) is difficult to apply to data when the ion concentration in the plant is much higher than that existing in the nutrient solution. The concentration of any given free ion in an absorbing cell is probably not higher than the concentration in the nutrient solution surrounding

[3] The papers by FLINT (3) are extremely interesting regarding H^+ and OH^- ions. The validity of his hypothesis is recognized. H^+ and OH^- ions are used for positive and negative charges in the present paper.

it. Chemical data which show a higher concentration of nitrate in a composite sample of tissues than is present in the solution surrounding the roots does not prove that a DONNAN equilibrium does not exist in the root hairs. The higher concentration in the composite sample may be accounted for by the ions associated with soluble or insoluble organic materials in cells other than those that actually absorb the nutrients. Furthermore, it is difficult to interpret data obtained from a mass of plant tissue in terms of the concentration in the nutrient solution. The DONNAN equilibrium hypothesis must be applied to those root-hair cells where absorption takes place and not to those cells much farther removed from the absorbing region. In tomato, in which nitrate occurs throughout the plant, much higher concentrations are recorded (11) than in apple (21) or asparagus (10), in which it is seldom found except in the fine roots. It is probably safe to assume that a DONNAN equilibrium does exist with respect to cells instrumental in absorbing ions from the nutrient solution.

On the basis of the data presented it would seem that a logical explanation for the slow recovery of ammonium and nitrate ions from plant extracts containing those ions is available. During the aspiration for the recovery of ammonium ions, the pH remains above 10, establishing negative charges on most of the amino acids present. These charges would readily be neutralized by some ammonium ions and other cations. Ammonium ions which are combined as mineral salts would be easily removed by aspiration, but those associated with soluble ampholytes would not be removed any faster than equilibrium could be re-established through replacement of ammonium by sodium ions after the free ions were removed. This would be a slow process.

The determination of nitrate nitrogen should be a more rapid process if the resulting ammonium ions were removed as soon as they are formed, because most of the nitrate ions probably exist as free or inorganic-salt ions. However, the aspiration process usually is much slower, so that equilibrium is established between the ammonium ions reduced from nitrate and the soluble ampholytes before the ammonium ions are removed from the solution. The determination of nitrate would then involve the same association and dissociation phenomena attendant to the determination of ammonium nitrogen. The greatest difference in the amounts of ions obtained by extraction with water and by electrodialysis occurs at those pH values farthest from the isoelectric points; the least difference is found when the greatest volume of ampholytic material is at its isoelectric point. In the case of tomato tissue the latter is around pH 6.0 to 6.5.

The explanation, therefore, for the retention of ions in cells in higher concentrations than that present in the nutrient solution seems comparatively simple. If we assume that the pH of the protoplasm of any given cell is the algebraic sum of all positive and negative charges where mineral anions and cations are in equilibrium with one another as well as with soluble organic anions and cations or with dissociated charges on cell colloids, a further assumption is necessary to explain the change in acidity of a nutrient solution as ions are absorbed. On the basis of the DONNAN equilibrium hypothesis, we may assume that there is a continual exchange of free ions through the cell membrane. The nitrate and ammonium ions must then play a role in changing the equilibrium in the cell by

means of the chemical changes attendant to the formation of amino acids with soluble carbohydrates, because it has been shown that changes occur in the equilibrium of the nutrient solution only when nitrate and ammonium ions are being assimilated (**22**). NIGHTINGALE (by letter) suggested that changes in pH occur at the surface of the cell membrane, even though a constant pH is maintained in the nutrient solution. In other words, if nitrate or ammonium ions are being rapidly assimilated (amino acids synthesized), the ions with which they were associated remain at the cell membrane and cause a temporary change in acidity, the degree depending on the rapidity with which excreted ions diffuse and come into equilibrium with the other ions in the solution. In water cultures this would be much more rapid than in sand cultures, which, in turn, would facilitate more rapid diffusion than in a soil. The fact that the ions associated with the nitrate or ammonium ions do not enter the cell as rapidly seems indirect proof that a DONNAN equilibrium phenomenon does exist—the concentration of free plus dissociable ions in the cell probably is never greater than in the nutrient solution.

It would seem that it is necessary to attribute another function to absorbed ions. From the results obtained by MATTSON (**7**) and evidence by the above data, certain ions have the function of keeping the protoplasm in a hydrated or highly dispersed condition. Cells of meristematic root tissue have a dense, hyaline protoplasm more or less uniform in appearance, containing the nucleus and occasionally other bodies of greater density. Proteins, which are insoluble organic complexes at their isoelectric points, and abundant in rapidly dividing cells, seem to be hyaline in appearance except when they are colored by pigments. In rapidly dividing cells they are probably in a highly dispersed condition.

ECKERSON (**2**) stated that parenchymatous cells of this nature have pH values as high as 7.6. Casein and soybean-seed protein become so highly dispersed in the presence of ammonium hydroxide at pH values above 6.5 that they cannot be separated from water with ordinary filters. The same proteins flocculate at pH 4.6 to 4.7—the pH of their isoelectric points—and are then readily separated from water by filtration. PEARSALL and EWING (**14**) stated that many vegetable proteins have isoelectric points between pH 3.0 and 5.6. ECKERSON (**2**) reported that xylem tissue is acid (pH 4.4) and that mature cells in which the protein may be precipitated are acid. In other words, the proteins are near the pH values of their isoelectric points and are no longer dispersed in the cells because of the acid reaction.

Cells which are primarily concerned in the assimilation of nitrate and ammonium ions, therefore, have saps with comparatively high pH values and viscous, highly dispersed protoplasts. The abundant protoplasm in the cells of phloem, cambium, other meristems, parenchyma, and root hairs consists of highly dispersed proteins in which there are many soluble amino acids, electrolytes, sugars, fatty materials, and other substances, some of which have amphoteric properties. One would expect a high degree of association between the ammonium and nitrate ions and the proteins in such a highly dispersed system.

When plants become "hard"—high in carbohydrates—many of these hyaline, rapidly dividing cells mature very quickly, and the composite pH of the tissue therefore becomes more acid (**2**), probably because of the presence of organic acids. The proteins become precipitated

and give the protoplasts a stringy-granulated appearance. It has been shown that tomato plants in this "hard" condition become very succulent soon after a nutrient solution containing ammonium is supplied, much more so than when nitrate is supplied (**20**). This would seem to be strong evidence that organic acids combine with the absorbed ammonium ions (a slower process with nitrate ions because they must first be changed to ammonium). The pH values of the cells are raised, causing the protoplasts to become dispersed and amino acids to be formed. This is soon followed by cell division, and evidence of growth is seen.

The dispersion of the proteins, therefore, probably results from combination with cations absorbed from the nutrient solutions. It has been shown (**23**) that sodium is very efficient in increasing the water-holding capacity of plant tissue and that calcium tends to counteract the effect of sodium. Many of these cations probably do not exist as free ions, as suggested by HOAGLAND, quoted by THERON (**19**), but as dissociation products of colloidal compounds (proteins) and amino acids. The degree of dispersion in a cell with a sap at pH 7.0 is then the result of the ratio of the various cations. MATTSON (**7**) also has shown that calcium suppresses the dispersion, particularly as the majority of the proteins may be considered electronegative. MATTSON further showed that iron and aluminum precipitate proteins and form undissociated complexes. These complexes have isoelectric points at pH values between the pH 4.0 of protein and pH 8.0 of the iron salt employed (in this case no aluminum was present in the nutrient solutions); the actual value depended on the proportion of protein to iron salt in combination. This may have some bearing on the difference in iron assimilation observed between nitrate-and ammonium-supplied trees in an experiment in which the effect of pH on nitrate and ammonium assimilation (**21**) was studied.

The high degree of dispersion or swelling of proteins with cations indicates a negative charged colloid of high intensity. These proteins are then able to combine with amino acids and electrolytes. The complex formed would have negative and positive charges which would be exchanged for other anions and cations. The pH of a cell would then be the point at which equilibrium of the dissociating negative and positive charges was established. The absorption of nitrate and ammonium and other ions would depend on the number of negative and positive charges which could be displayed at any given pH. The number of free ions in the cell would depend on the concentration in the nutrient solution, while some are combined with ampholytes in the cell. The pH of a cell may then be considered as resulting from exchange reactions with reference to the absorption of anions and cations. That there would be considerable overlapping is graphically shown in figure 1. This overlapping of absorption of cations and anions is the result of the ratio of dissociation of negative and positive charges, depending on the relation of the pH to the nutrient solution and the pH of the isoelectric points of the various ampholytes present. Theoretically, cation or anion absorption would not cease until the pH of the nutrient solution was more acid or more alkaline than the pH of the lowest or highest isoelectric points of the constituent parts that make up the protoplast. Fortunately, these conditions seldom occur under normal conditions, because the protein would become coaglated or so highly dispersed that life could not exist. PIRSCHLE (**15**), using nitrate nitrogen, obtained a bimodal

curve for growth of some plants over the pH range from 3.0 to 9.0. He found less vigorous growth in many plants at pH 6.0 than at pH 4.0 or 8.0. This may have been related to the ampholytic nature of protoplasm.

RABER (16) assumed that the so-called "cell membrane" is negatively charged. The cell membrane of an absorbing cell, however, is probably in equilibrium with its protoplast and may carry negative or positive charges, depending on the pH of the nutrient solution and upon the nature of the material at the periphery of the cell. If the membrane or material has amphoteric properties, it probably carries either one or the other charge, depending on the relation between the pH of the isoelectric point and the pH of the nutrient solution. This would seem reasonable, in view of the fact that both ions must be absorbed in order to account for the growth PIRSCHLE obtained at different pH values. It must be remembered that the absorbing area of feeding roots is the newly formed region back of the tip where root hairs are abundant. Any effect of a nutrient solution on the assimilation of ammonium or nitrate ions is exerted through these root hairs, especially in the apple, in which the assimilation of nitrate ions to amino acids occurs in the very fine roots (21).

The synthesis of amino acids in the roots of apple, accompanied by utilization of carbohydrates, undoubtedly causes changes in the pH of the cell contents, which helps to account for the diurnal variations, observed by INGALLS and SHIVE (4), in the pH of the composite plant tissue. THERON (19) has shown that the pH of the nutrient solution also affects the buffer capacity of the protoplasts of the root cells quite materially. These changes in the pH of the cells therefore account for a system that is shifting back and forth, in which the ratio of cations to anions that displace positive and negative charges is continually changing. This change in ratio of charges and the effect of the anions and cations thus combined on the dispersion of the protoplast and the saponification of fats undoubtedly influence markedly the growth and division of cells and the assimilation of ammonium and nitrate ions. These processes are probably most favored just above or below the normal pH of the cell sap. The rate at which the two ions are assimilated then depends on the reduction of the nitrate ions. If more ammonium ions are being absorbed by one plant than are made available by the reduction of nitrate ions in another plant, amino acids will accumulate more rapidly in the former, providing, of course, that carbohydrates are plentiful.

From this discussion it may be assumed that any method of removing anions or cations from living tissues which does not take into consideration the isoelectric points of proteins and amino acids would fall far short of giving more than comparative data. For complete recovery the ions must be disposed of by electrical devices which remove them as soon as they are released through equilibrium phenomena, or the tissue must be completely destroyed so that the ions are released to a free state. This discussion may throw some light on the difficulties encountered in obtaining data which agree or check according to theoretical concepts.

Summary

Data are presented which show that nitrate and ammonium ions may not be recovered from living tissue in absolute amounts. This results from the fact that protoplasm contains proteins and amino

acids which have amphoteric properties and which form dissociation compounds with nitrate and ammonium ions which obey the laws of electrochemistry. The conditions in cells necessary to support the above assumption are suggested, together with an explanation of conditions which favor nitrogen assimilation and subsequent growth in plants.

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PIGMENT GLANDS OF COTTONSEED. III. DISTRIBUTION AND SOME PROPERTIES OF COTTONSEED PIGMENTS

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Introduction

For a number of years the direct solvent extraction of cottonseed for the production of oil and meal has been recommended as a practical method of processing (9, 10, 11, 13, 14, 19, 20, 24, 25), but it has only recently been applied on a commercial scale in the United States. The recent successful development of solvent extraction methods for the processing of soybeans (12), as well as the current trend toward the use of processes requiring the employment of a minimum of labor, has intensified interest in the application of this method to cottonseed. However, the unique system of pigments in the kernel of the cottonseed not only differentiates this seed from others but poses problems which are not encountered in the processing of other commercial oilseeds. Although the control of color is not the only difficulty, it is one of the principal ones encountered in application of solvent extraction to cottonseed on an industrial scale.

It has been shown (4) that the pigment glands, which contain most of the pigments of the kernel, are mechanically very strong and are resistant to the action of a considerable number of types of organic solvents such as the hydrocarbons, chlorinated hydrocarbons, and glycerides. These glands possess a density of approximately 1.378 gm./cc., which is less than that of the other tissue of this seed. On the basis of these properties a method which employs mechanical disintegration and flotation was devised as a means of separating pigment glands from other seed tissue and oil.

It has also been shown (6) that the pigment glands owe their mechanical strength to a thick encompassing wall, composed of cellulose impregnated with a pectinaceous material and exteriorly coated with a thick layer of cutin. This wall is readily ruptured by contact with water and a few water-miscible alcohols, ketones, and ethers of low molecular weight. The relatively slow rupturing of the wall on contact with other organic liquids was found to result from the presence of moisture in the seed or solvent.

Even casual examination of a section of cottonseed will reveal that most of the deeply colored pigments of the kernel are concentrated in the pigment glands. The oil and extraglandular tissue are colored a faint yellow, whereas the glands may vary from yellow, through various shades of orange and red, to dark purple. It has generally been assumed that all the gossypol and most of the other pigments of the seed are segregated in the glands, but no accurate determination has heretofore been made of the extent to which this occurs or to differentiate the extra- from the intraglandular pigmented material. Consequently, the present investigation was undertaken with the object of ascertaining the distribution of the cottonseed pigments between the glands and the extraglandular tissue and oil. Further information has also been sought concerning the behavior of the pigments

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during extraction of the oil with different solvents.

Material and methods

PREPARATION OF SEED, MEAL, AND GLAND SAMPLES FOR EXTRACTION.—Samples of seed kernels were finely divided by flaking or grinding to insure subsequent contact of the pigment glands with the solvent. When small samples of seed were to be extracted, the kernels were ground in a Bauer mill or in a glass mortar and then passed through a U.S. No. 50 sieve. Larger samples were prepared by flaking the kernels on rolls set at 0.003 in. clearance; this produced flakes varying in thickness from 0.004 to 0.008 of an inch.

Samples of defatted meal were prepared in a Soxhlet apparatus by extracting weighed amounts of flaked kernels with petroleum naphtha[2] for 4–6 hours.

Samples of pigment glands and of gland-free meal were prepared by means of the previously described flotation process (**4, 23**). Some of the samples were freed of adhering tissue by prolonged agitation of flaked meats in the flotation mixture (**4**); others were freed of small residual amounts by shaking them with finely divided silica suspended in petroleum naphtha (**23**).

Samples of flaked or ground meats, of defatted meal, or of glands which had been wetted to produce preliminary rupturing were dried for 18 hours in a vacuum desiccator over anhydrous calcium sulfate before extraction with organic solvents.

PREPARATION OF EXTRACTS.—Small samples of seed, of defatted meal, or of pigment glands were extracted with chloroform, diethyl ether, or petroleum naphtha under the conditions previously described (**2, 5**) as being essential to insure complete extraction and subsequent stability of the pigments; that is, by equilibration for periods of not less than 24 hours at 38° F., and in the absence of light. All extracts were stored under these same conditions, and their absorption spectra were determined within a period of 72 hours after the extraction was started. Extraction of pigments with aqueous solutions of water-miscible organic solvents was accomplished by equilibration for periods of 10–30 minutes.

Complete extraction of the residual gossypol from solvent-extracted meals, and from meals freed of pigment glands by the flotation method, was accomplished by the following adaptation of a recently published method (**22**) for the determination of gossypol in cottonseed and cooked cottonseed meal. To a weighed sample of meal, ground to pass a U.S. No. 50 sieve, there was added 30 ml. of 30% aqueous ethanol (by weight). The mixture of meal and solvent was shaken vigorously and allowed to stand for one-half hour. In order to dissolve the resultant suspension of gossypol, 70 ml. of 72% aqueous ethanol (by weight) was added. The mixture was then agitated and centrifuged. Alternatively, samples of gland-free meal were treated directly with 60% aqueous ethanol. Aliquots of the supernatant solution were transferred to chloroform for application of the alkaline extraction method (**5**) for the isolation of gossypol, followed by the antimony trichloride spectrophotometric method (**3**) for the determination of the concentration of the isolated gossypol.

The size of meal sample used in preparation of the aqueous ethanol extract was adjusted on the basis of its gossypol content. Since the gossypol content of meals

[2] The petroleum naphtha used in this investigation was a commercial pentane-hexane fraction, boiling range 95°–138° F.

extracted with petroleum naphtha varied from 0.5 to 2.0%, relatively small samples (0.25–1.0 gm.) of these meals were used. Larger samples (2.0–10.0 gm.) of diethyl ether-extracted and gland-free meals were used because of their low content of gossypol.

DETERMINATION OF ABSORPTION SPECTRA AND PIGMENT CONTENT.—All absorption spectra were measured with a Beckmann quartz spectrophotometer except those of the antimony trichloride reaction products, which were read in a Coleman monochromator spectrophotometer. Extinction coefficients of extracts were expressed as $E^{1\%}_{1\text{ cm.}}$ or as $E^{\text{gm./l.}}_{1\text{ cm.}}$ in terms of the weight of kernels, glands, or meals extracted. The extinction coefficients of extracted oils were expressed as $E^{1\%}_{1\text{ cm.}}$ in terms of the weight of solvent-free oil dissolved.

All absorption measurements were made on chloroform solutions to eliminate differences resulting from the effect of the solvent. The absorption spectra of extracts prepared with chloroform were read directly, those for solvent-free extracted oils after solution of the oils in chloroform, and those for extracts prepared with solvents other than chloroform after transfer to chloroform. In order to transfer the pigments from water-immiscible solvents to chloroform, aliquots of the extracts were evaporated under reduced pressure at room temperature, and the residues were dissolved in measured volumes of chloroform. When a water-miscible solvent was used for extraction, a measured volume of chloroform was added to an aliquot of the extract, after which water was added to the mixture until all the color had been transferred to the chloroform layer upon shaking and centrifuging the mixture.

The gossypol content of seed was determined by direct application of the antimony trichloride spectrophotometric method (2) to chloroform solutions of the extracts. For the determination of the content of residual gossypol of solvent-extracted meals and of meals freed of pigment glands by the flotation process, gossypol was first isolated from the extracts by the alkaline extraction method (5); the antimony trichloride spectrophotometric method was applied to the isolated gossypol. Preliminary isolation of gossypol by the alkaline extraction method was also applied for the accurate determination of the gossypol content of petroleum naphtha-extracted oils.

The gossypurpurin content of seed, meal, and gland samples was estimated on the basis of the extinction coefficients of chloroform extracts at 564–567 mμ, the wave-length region of maximum absorption of gossypurpurin (1, 5). The percentage concentration was calculated by means of the formula ($E^{1\%}_{1\text{ cm.}}$ extract/$E^{1\%}_{1\text{ cm.}}$ gossypurpurin) × 100.

Results

INTRAGLANDULAR PIGMENTS.—Anhydrous petroleum naphtha was used for extracting the oil and the oil-soluble extraglandular material from dried cottonseed. The absence of both gossypol and gossypurpurin was demonstrated by spectrophotometric examination of the extract; this constitutes evidence that all the gossypol and gossypurpurin of the seed kernel are segregated in the pigment glands.

Gossypol constituted from 39 to 49% and gossypurpurin from 0.61 to 1.73% of the weight of glands separated from several samples of pure-bred seed of different varieties of *Gossypium hirsutum* (table 1). Since all the gossypol and gossypurpurin was segregated in the glands, the gland content of the kernels was determined on the basis of the rela-

tive content of gossypol in the separated glands and in the corresponding kernels. The percentage, by weight of glands, in each sample was calculated by means of the formula: (% gossypol in seed/% gossypol in glands) × 100. Because of the instability of gossypurpurin in solution, similar calculations based on the ratio of this pigment in the seed and in the corresponding samples of glands were considered to furnish a less accurate measure of the content of glands. The content of glands in different samples of seed was found to vary within wide limits (2.37–4.81%).

The spectral absorption resulting from the presence of gossypol in the aforementioned samples of pigment glands (table 1) was calculated throughout the wave-length region of 250–430 mμ—the region of characteristic selective absorption by this pigment—by dividing the extinction coefficient for pure gossypol (7) at each wave length by the gossypol content of the glands. The curve constructed with these values (typical example in fig. 1, curve *B*) was identical, within the limits of experimental error, with the experimentally determined curve of the absorption spectrum (typical example in fig. 1, curve *A*) of the chloroform extract of the glands, thus demonstrating the absence of any detectable amounts of a yellow pigment other than gossypol in the glands examined.

Corresponding samples of glands were extracted with chloroform, diethyl ether, methanol, ethanol, and aqueous mixtures of the last two solvents. The absorption spectra of the chloroform solutions of these extracts were identical with one another and with the spectral curve calculated for gossypol (fig. 1, curve *B*) throughout the region from 250 to 430 mμ.

The complete absorption spectra from 240 to 600 mμ of chloroform extracts of pigment glands (typical example in fig. 1, curve *F*) were almost identical with the composite curves obtained by plotting the values calculated for both gossypol and gossypurpurin in the extracts, thus indicating that these pigments were the only ones present in detectable amounts in the glands examined.

TABLE 1

COMPARISON OF GOSSYPOL AND GOSSYPURPURIN CONTENT OF SEED KERNELS AND OF CORRESPONDING PIGMENT GLANDS

Nature and variety of products	Gossypol content (%)*	Gossypurpurin content (%)†	Content of glands in seed kernels (%)‡
1. Delfos 651§	1.09	0.055	2.66
2. Pigment glands from (1)	40.9	1.73	.....
3. Cleve wilt‖	2.39	0.0388	4.81
4. Pigment glands from (3)	49.2	0.612	.....
5. D & P L-45¶	1.14	0.0368	2.92
6. Pigment glands from (5)	39.0	1.08	.....
7. Delfos-451-42-43**	1.11	0.0286	2.37
8. Pigment glands from (7)	43.96	1.08	.....

* Determined by antimony trichloride spectrophotometric method.

† Calculated on basis of $E^{1\%}_{1cm.}$ of chloroform extract and $E^{1\%}_{1cm.}$ of pure gossypurpurin, 225.7, at 565–566 mμ.

‡ Calculated on basis of gossypol content of glands and corresponding seed: (% gossypol in seed/% gossypol in glands)–×100.

§ Pure-bred cottonseed from 1943 crop at Stoneville, Mississippi, stored for 2 years prior to separation of glands.

‖ Pure-bred cottonseed from 1943 crop at Clemson, South Carolina, stored for 2 years prior to separation of glands.

¶ Pure-bred cottonseed from 1944 crop at Stoneville, Mississippi, stored for 2½ years prior to separation of glands.

** Pure-bred cottonseed from 1945 crop at Stoneville, Mississippi, stored for 1½ years prior to separation of glands.

Aqueous alkali extracted all the color from chloroform extracts of pigment glands. The material recovered by acidification of the alkaline extract gave an antimony trichloride reaction product which was stable for 24 hours and exhibited the absorption spectrum charac-

teristic of the reaction product of pure gossypol.

EXTRAGLANDULAR OIL-SOLUBLE PIGMENTS.—The extinction coefficients for gossypol in each sample of seed kernels investigated were calculated by dividing the value of $E_{1\text{ cm.}}^{\text{gm./l.}}$ for pure gossypol at each wave length throughout the range from 250 to 430 mμ by the gossypol content of the seed. The curves constructed by plotting the values calculated for gossypol in the seed extracts (typical example in fig. 1, curve *D*) were significantly lower than the experimentally determined curves of the absorption spectra of the corresponding extracts of the seed (typical example in fig. 1, curve *C*).

Anhydrous light petroleum naphtha was used with dried cottonseed flakes to extract the extraglandular, oil-soluble material, free of intraglandular material. The absorption spectrum of the extract (fig. 2, curve *B*) exhibited a broad maximum at 368–374 mμ and an inflection at 280 mμ, in contrast to the maxima observed at 362–366, 288–289, and 278–280 mμ for gossypol in chloroform ex-

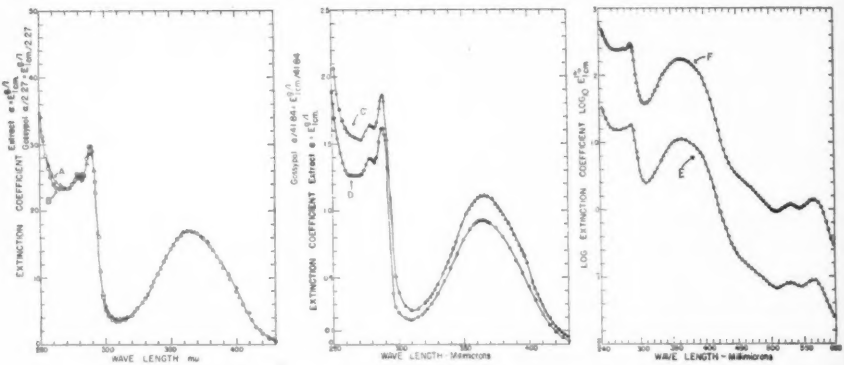

FIG. 1.—Absorption spectra of (*A*) chloroform extract of cottonseed pigment glands, (*B*) gossypol in extract of pigment glands, (*C*) chloroform extract of corresponding kernels, (*D*) gossypol in extract of kernels, (*E*) chloroform extract of corresponding kernels, and (*F*) chloroform extract of pigment glands.

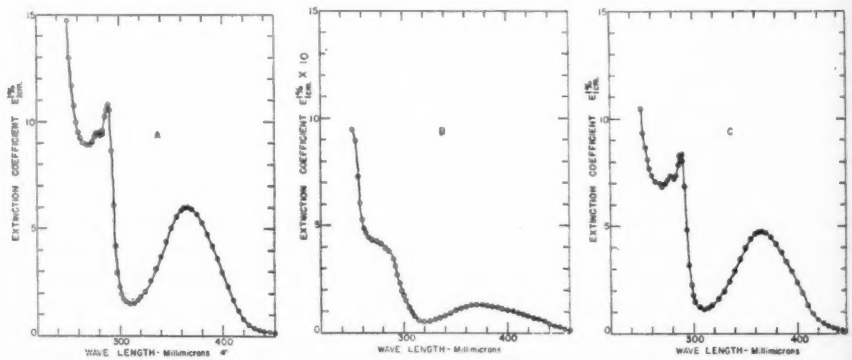

FIG. 2.—Absorption spectra of (*A*) chloroform extract of cottonseed, (*B*) petroleum naphtha extract of same seed, (*C*) chloroform extract of defatted seed.

tracts of the same seed (fig. 2, curve *A*) and of defatted seed (fig. 2, curve *C*).

When special precautions were not taken to reduce the moisture content of seed and solvent, or when extraction was prolonged, or both, some of the glands were ruptured during extraction with petroleum naphtha. Consequently, the absorption spectra of such extracts (fig. 3, curve *A*) indicated the presence of both gossypol and extraglandular pigment. Extraction with aqueous alkali was used to remove gossypol and the decomposition product of gossypurpurin from the petroleum naphtha extract. The absorption spectrum obtained with the residual solution (fig. 3, curve *B*) was the same as that obtained with a petroleum naphtha extract of dry seed (fig. 2, curve *B*).

The amount of gossypol in the original petroleum naphtha extract of moist seed was 0.336% of the weight of extracted oil. The composite curve, based upon the sum of the absorption calculated for gossypol in the extract and the absorption of the residual extract, was slightly lower than that of the original extract. It is not known whether this difference is a result of a shift in the absorption spectrum of the extraglandular pigment in the presence of alkali, or whether it is caused by the removal of a small amount of the extraglandular material during alkaline extraction of gossypol from the extract.

The extraglandular material formed an unstable reaction product with antimony trichloride which exhibited some selective absorption in the visible and near ultraviolet wave-length region which increased in intensity toward the shorter wave lengths. Transitory absorption maxima were usually observed at 420, 450–460, and 490 mμ. The absorption was intensified as the reaction product aged, the increase being greater toward the regions of shorter wave lengths.

PIGMENTATION OF SOLVENT-EXTRACTED COTTONSEED MEAL.—All the gossypol was extracted from cottonseed of moderate moisture content during contact for a period of 24 hours with commercial-grade chloroform containing

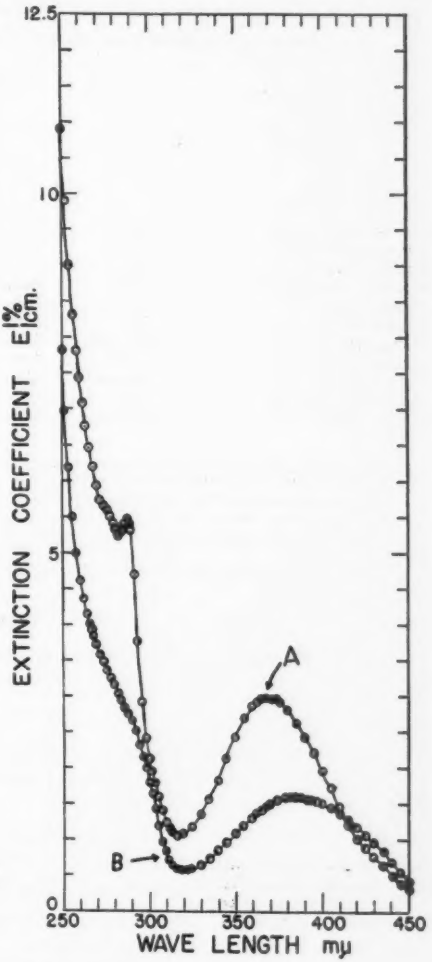

FIG. 3.—Absorption spectra of (*A*) petroleum naphtha extract of moist cottonseed and (*B*) same extract after removal of gossypol by extraction with aqueous alkali.

some moisture (table 2). Dried chloroform extracted only part of the total gossypol present in dried cottonseed during the same period of contact. After preliminary moistening of the seed to rupture the pigment glands, all the gossypol was extracted within a few minutes by chloroform or aqueous ethanol.

Light petroleum naphtha, in which gossypol is very slightly soluble, extracted only a fraction of the total gossypol from cottonseed (table 3), even after preliminary moistening of the seed to rupture the glands. Since dried petroleum naphtha extracted some gossypol from dried flakes, it was apparent that

TABLE 2

EFFECT OF MOISTURE ON EXTRACTION OF GOSSYPOL BY ORGANIC SOLVENTS

Sample	Treatment	Method of extraction			Gossypol extracted (% of total)*
		Preliminary treatment	Solvent	Time of contact (hours)	
Cottonseed (CS-165)	None	None	Chloroform	24	100
Cottonseed (CS-165)	Dried†	Dried‡	Chloroform	24	40.8
Cottonseed (CS-165)	None	Ethanol (30%)§	Ethanol (60%)	$\frac{1}{6}$	100
Pigment glands (C-78)	None	None	Chloroform	24	100
Pigment glands (C-78)	Dried†	Dried‡	Chloroform	24	28.6
Cottonseed (CS-101)	None	None	Chloroform	2	100
Cottonseed (CS-101)	Wetted‖	None	Chloroform	5	100
Cottonseed (CS-926)	None	None	Chloroform	24	100
Cottonseed (CS-926)	Wetted‖	None	Chloroform	20	97
Cottonseed (CS-103)	None	None	Chloroform	24	100
Cottonseed (CS-103)	Wetted‖	None	Chloroform	$\frac{1}{60}$	90

* Total gossypol determined by equilibration of untreated seed with chloroform at 38° F. for 24 hours and application of antimony trichloride spectrophotometric method to extract.

† Dried in vacuum desiccator over anhydrous calcium sulfate.

‡ Dried by treatment with calcium chloride.

§ Treated with 30% ethanol (30% ethanol by weight), then 72% ethanol added to yield a final extract of 60% (by weight) ethanol.

‖ Flaked or ground seed thoroughly moistened, then dried overnight in vacuum desiccator over anhydrous calcium sulfate.

TABLE 3

EXTRACTION OF GOSSYPOL FROM COTTONSEED BY PETROLEUM NAPHTHA

Preparation of seed for extraction	Treatment of solvent	Conditions of extraction		Gossypol extracted (% of total)*
		Time (hours)	Temperature (° F.)	
Ground meats, wetted, then dried†	None	$\frac{1}{20}$	76	58
Seed hulled and flaked‡	None	2	76	32
Flaked meats, dried†	Dried§	24	38	12
Seed dried,†‖ hulled, and ground	Dried§	24	38	0

* Gossypol determined on basis of antimony trichloride reaction of chloroform extracts of original seed samples and of petroleum naphtha extracts.

† Dried in vacuum desiccator over anhydrous calcium sulfate for 24 hours.

‡ Moisture content of flaked meats, 8.44%.

§ Dried by treatment with calcium chloride.

‖ Moisture content of dried seed, 3.85%.

some of the pigment glands had been ruptured during flaking of the seed. Drying of the seed before flaking prevented extraction of gossypol by anhydrous petroleum naphtha.

As another means of comparing the effectiveness of different organic solvents for the extraction of gossypol from cottonseed, the gossypol content of the different solvent-extracted meals was determined. It was thus found that methanol, ethanol, isopropanol, acetone, 1,4-dioxane, and aqueous mixtures of these solvents extracted all the gossypol from cottonseed. Extraction of cottonseed with diethyl ether, or removal of pigment glands from the seed by the flotation method, removed all but traces of gossypol (table 4).

TABLE 4

GOSSYPOL CONTENT OF SOLVENT-EXTRACTED COTTONSEED*

Seed	Solvent	Time of contact (hours)	Gossypol content of meal (%)†
CS-527‡	Commercial hexane and tetrachlorethylene§	24	<0.0058‖
CS-527	Diethyl ether	24	0.025
CS-527	Petroleum naphtha	6	0.859
CS-527	Commercial hexane¶	6	1.91
1610-E-22	Commercial hexane and tetrachlorethylene§	24	<0.007‖
CS-165	Commercial hexane and tetrachlorethylene§	24	<0.022‖
1619-E-6	Commercial hexane and tetrachlorethylene§	24	<0.030‖
1619-E-17	Commercial hexane and tetrachlorethylene§	24	0.00**
1630-E-18	Diethyl ether	8	<0.032‖
1630-E-13	Diethyl ether	8	<0.032‖
1630-E-17	Diethyl ether	8	<0.052‖
486-E-77	Commercial hexane¶	6	0.80
486-E-27	Commercial hexane¶	6	1.90
CS-165	Petroleum naphtha	2	1.81

* Commercial grade solvents and seed of moderate moisture content were used.

† Extracted with 30% aqueous ethanol (by weight) followed by 72% aqueous ethanol, giving a final mixture of 60% ethanol; gossypol then determined by antimony trichloride spectrophotometric method.

‡ Moisture reduced to 5%.

§ Pigment glands removed by gland flotation process using mixture of commercial hexane and tetrachlorethylene having a density of 1.378 gm./ml.

‖ Some absorption at 520 mμ owing to reaction of interfering substances with antimony trichloride.

¶ Boiling range 146°–158° F.

** Determined by application of antimony trichloride spectrophotometric method to aqueous alkaline extract of chloroform solution of aqueous ethanol extract of meal.

The variation in content of gossypol observed in different samples of cottonseed meal extracted with petroleum naphtha (table 4) demonstrates the variety of conditions—namely, moisture content of seed and solvent and duration and temperature of extraction—which determines the amount of gossypol removed from cottonseed by such extraction. Microscopic examination of these meals showed considerable variation in the distribution of pigments remaining after extraction with this solvent. Most of the meals defatted with petroleum naphtha contained a large number of intact pigment glands. In many cases, however, the extraglandular tissue was colored a distinct yellow, while in others the tissue was almost colorless. It was thus evident that the yellow meals contained much of the pigments which had been discharged from ruptured pigment glands and had not subsequently dissolved in the petroleum naphtha during extraction.

EXTRACTION OF RESIDUAL PIGMENTS FROM DEFATTED MEAL.—As shown by the first two entries in table 5, and as previously established (**2**), independent extractions of defatted cottonseed meal with chloroform frequently do not yield reproducible results. Since preliminary moistening of the defatted meals, in order to rupture the pigment glands, was found to reduce the amount of gossypol extractable with chloroform or diethyl ether (table 5), it was apparent that the resistance of the gland walls was not solely responsible for the difficulty encountered in removing the residual pigments from defatted meals by extraction with these solvents. After preliminary rupture of the gland walls by the use of water or aqueous ethanol mixtures of high water content, the gossypol was completely and rapidly extractable with ethanol. The residual meals were almost completely colorless except those which had contained relatively large amounts of gossypurpurin. Treatment with methanol, isopropanol, acetone, or 1,4-dioxane was also found to remove all the gossypol from defatted meal. Only the two last-mentioned solvents were effective in removing all the residual gossypurpurin or its yellow decomposition product from defatted cottonseed.

TABLE 5

EFFECT OF MOISTURE ON EXTRACTION OF GOSSYPOL FROM COTTONSEED MEAL AFTER PRELIMINARY DEFATTING WITH PETROLEUM NAPHTHA

Meal	Treatment before final extraction	Solvent for extraction	Gossypol (%)*
C-101....	None	Chloroform	0.65
C-101....	None	Chloroform	0.35
C-101....	Wetted†	Chloroform	0.17
C-926....	None	Chloroform	1.00
C-926....	None	Diethyl ether	1.09
C-926....	Wetted†	Chloroform	0.29
C-926....	Wetted†	Diethyl ether	0.39
CS-165...	Ethanol(30%)‡	Ethanol (60%)	1.81
CS-165...	None	Chloroform	1.48
CS-165...	Wetted†	Chloroform	0.44
CS-165...	Wetted†	Ethanol (60%)	1.23
CS-56....	Ethanol(30%)‡	Ethanol (60%)	0.85
CS-56....	Wetted†	Chloroform	0.01
CS-527...	Ethanol(30%)‡	Ethanol (60%)	1.92
CS-527...	None	Chloroform	1.38
CS-610...	Ethanol(30%)‡	Ethanol (60%)	0.86
CS-610...	None	Chloroform	0.86

* Gossypol content of extract determined by antimony trichloride spectrophotometric method; values expressed on basis of weight of meal.

† Meal thoroughly moistened, then dried overnight in vacuum desiccator over anhydrous calcium sulfate.

‡ Treated with 30% aqueous ethanol (30% ethanol by weight), then 72% aqueous ethanol added to yield final extract of 60% ethanol.

Discussion

Application of the recently developed flotation process (**4, 23**) for separation of intact pigment glands from other parts of the seed has provided material for the determination of the content of pigments of the glands separated from four samples of different varieties of *Gossypium hirsutum*. Gossypol was found to constitute from 39.0 to 49.2%, and gossypurpurin from 0.612 to 1.73%, of the weight of the separated glands. The demonstration that all the gossypol and gossypurpurin of the kernel is concentrated in the pigment glands has made possible accurate calculation of the average weight of glands in each sample of seed investigated. The average content of glands was found to vary from 2.37 to 2.92% of the weight of the seed containing smaller amounts of gossypol and was 4.81% of the weight of the seed containing the largest amount. Thus, a general correlation has been established between the content of gossypol and of glands in the seed. Since these seed lots were grown under different environmental conditions and were stored for different periods of time, it would appear that the amount of

gossypol in the glands is relatively constant; the gossypol content of the seed is, therefore, largely determined by the content of glands. The variation in the gossypurpurin content of glands separated from different samples of cottonseed is consistent with PODOL'SKAYA's (16, 17) observation that this pigment can be detected only in maturing and stored seed and that it varies greatly during these periods.

SMIRNOVA (21) has reported that genetic factors are more important than environmental factors in determining both the average number of glands and the content of gossypol in the cottonseed kernel. Seed from different varieties of *G. herbaceum* grown under different conditions contained only a small number of glands and very little gossypol. Seed of different varieties of *G. hirsutum* were intermediate in the average number of glands and in gossypol content, while seed from different varieties of *G. barbadense* contained the largest number of glands and the largest amount of gossypol.

The seed lots examined during the present investigation were all of the same species, and the selection of varieties was too limited to permit differentiating between environmental and genetic factors. However, because of the wide variation in size of pigment glands in different parts of the kernel and in different samples of seed, more accurate correlations can be expected between the relative weights of glands and gossypol than between the number of glands and the weight of gossypol in the kernel, as proposed by SMIRNOVA.

Gossypol and gossypurpurin are the only pigments which could be detected in the extracts of the limited number of samples of pigment glands examined. The absence of these pigments in dry petroleum naphtha extracts of dry cottonseed indicated that they are segregated in the glands. Incontrovertible evidence for the presence of other pigments in the glands can be obtained only by their actual detection. In the absence of this direct evidence, however, deductions concerning the localization of other pigments can be based on indirect evidence concerning the relative difficulty with which the pigment is extracted from cottonseed. Gossyfulvin (3, 7) was not detected in any of the extracts of pigment glands examined but it does not appear to be of very frequent occurrence in cottonseed. Since prolonged contact with chloroform or ether has been reported (2) to be necessary for extraction of gossyfulvin from seed in which it occurs, it can be inferred that this pigment occurs in the glands rather than in the surrounding tissue. The observation that aqueous alkali extracts all the color from chloroform extracts of separated glands, coupled with the observed stability of the antimony trichloride reaction product of such extracts, provides reliable evidence that none of the yellow, oil-soluble, nonacidic pigment detected in cottonseed extracts occurs in the glands.

The absorption spectra of chloroform extracts, and of chloroform solutions of diethyl ether and aqueous ethanol extracts, of several samples of pigment glands separated from different lots of cottonseed were found to be identical with the absorption spectra calculated for gossypol in the extracts (fig. 1, curves *A*, *B*). Thus it would appear that the structure of gossypol is not altered during its extraction from the glands and subsequent purification. It can further be deduced that gossypol is not chemically combined with any of the other components of the glands but occurs as a colloidal, gelatinous suspension in the

glands. Because of the extreme instability of gossypurpurin in solvents other than chloroform, the absorption spectrum of the isolated pigment could be compared only with that of chloroform extracts of pigment glands.

The yellow extraglandular material is obtained free of intraglandular pigments by treating dry seed with dry petroleum naphtha; this procedure avoids rupture of the glands. Moreover, since the extraglandular pigment is nonacidic, it is not extracted from its solutions in organic solvents by treatment with aqueous alkali, whereas both gossypol and gossypurpurin, and the yellow decomposition product of gossypurpurin, are quantitatively extracted from such solutions. The absorption spectrum and chemical properties of this newly detected pigment differentiate it from gossypol, gossypurpurin, the yellow decomposition product of gossypurpurin, gossyfulvin, and the oil-soluble carotenoid pigment reported by PODOL'SKAYA (**15, 18**). Since this pigment is apparently stable in solution in the oil in contact with the reactive constituents of the seed tissue, it can be inferred that it is a relatively inert, nonpolar compound. Investigations (**8**) of its behavior in cottonseed oil extracted with petroleum naphtha indicate that it is essentially unaffected during alkali refining and subsequent bleaching of the oil. The carotenoid pigment reported by PODOL'SKAYA could not be detected in any cottonseed extract examined. However, since this pigment is readily extracted from cottonseed by petroleum naphtha, it can be inferred that in the seed in which it occurs it is found in solution in the oil surrounding the pigment glands.

Solvent-extracted cottonseed meals have been shown to fall into several categories with respect to the distribution and total content of pigments. Treatment of flaked seed with water-miscible alcohols, ketones, and ethers of low molecular weight, or with aqueous mixtures of any one of these solvents, ruptures the pigment glands. The pigment content of the residual meal is therefore determined entirely by the solubility of the pigments in the solvents or solvent mixtures. Acetone and dioxane extract all the pigments and yield almost colorless meals. Because of the very slight solubility of gossypurpurin and its yellow decomposition product in the alcohols, these solvents remove all the pigments with the oil only when the original seed contains relatively small amounts of gossypurpurin.

If moisture is rigorously excluded, the meals remaining after extraction of the oil with organic solvents, other than water-miscible alcohols, ketones, and ethers of low molecular weight, contain none of the oil-soluble extraglandular pigments, but most of the intraglandular pigments remain in intact pigment glands with the meal. With the use of moist solvents in which the pigments are soluble, such as commercial-grade chloroform and diethyl ether, the pigment glands are slowly ruptured and most of the pigments are removed from the meal during prolonged extraction. Preliminary wetting of the seed in order to rupture the pigment glands renders the pigments immediately extractable with the aforementioned solvents.

The extent to which the pigments are extracted from the seed by solvents in which they are not very soluble, such as the petroleum naphthas, will depend upon the conditions of extraction. When the pigment glands are ruptured by moisture in the seed before extraction of the oil, a relatively large proportion of the pigments will be dissolved in the oil and solvent mixture to which they are exposed during the initial stages of the extraction. Most of the pigments which are

discharged from pigment glands (ruptured during prolonged extraction and removal of most of the oil) are not removed by the extracting solvent but remain adsorbed on the meal. Consequently, meals obtained by extracting the oil with petroleum naphtha in the presence of moderate amounts of moisture will contain part of the pigments in the intact pigment glands and part will be adsorbed on the extraglandular tissue.

The adsorbed pigments can be removed from defatted cottonseed meal only by treatment with polar organic solvents. Treatment of such meals with alcohols or aqueous mixtures thereof removes all the adsorbed gossypol as well as that contained in the intact glands, but these solvents remove only small amounts of the yellow decomposition product of gossypurpurin. Meals which are essentially free of color were obtained by supplementary extraction of defatted meals with acetone or 1,4-dioxane, even when the original seed contained relatively large amounts of gossypurpurin.

Summary

1. All the gossypol and gossypurpurin of the cottonseed kernel are segregated in the pigment glands, and they constitute the only detectable pigments in the glands of four varieties of seed of *Gossypium hirsutum* which were examined.

2. The gossypol content of the glands is relatively constant, this pigment constituting up to approximately 50% of the weight of the glands, whereas the gossypurpurin content is relatively low and variable.

3. On the basis of the direct correlation observed between the amounts of glands and of gossypol in the kernels, it is suggested that the gland content is the principal factor which determines the gossypol content of the cottonseed kernel.

4. A yellow pigment has been detected in solution in the oil of the extraglandular tissue of the kernel. This pigment has been obtained free of gossypol, gossypurpurin, and the yellow decomposition product of gossypurpurin. Partial characterization of the extraglandular pigment has shown it to be relatively stable and to differ from every pigment previously detected in cottonseed.

5. On the basis of the distribution and properties of the pigments of cottonseed, methods for their extraction and estimation have been developed.

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INFLUENCE OF PHOTOPERIOD ON MICROSPOROGENESIS IN COSMOS SULPHUREUS CAV. VAR. KLONDIKE[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 590

GRACE C. MADSEN

Introduction

Degenerative cytological changes induced by environmental effects on microsporogenesis and upon the development of pollen grains have been reported by a number of investigators (**7, 8, 10, 12, 13, 14, 15, 16**).

Photoperiodic effects on microsporogenesis in Biloxi soybean were investigated by NIELSEN (**11**). Plants given 16- or 21-hour postinductive photoperiods following fewer than six photoinductive cycles failed to show development beyond the early prophase stages of meiosis. Those receiving six to ten photoinductive cycles showed degenerative changes, such as enlarged sporocytes with highly

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vacuolate cytoplasm, heavily staining sporogenous tissue, failure of telophasic reconstruction, degenerate microspores, and abnormal pollen.

In the classical investigations on photoperiod by GARNER and ALLARD, *Cosmos* was classed as a short-day plant (4). When sufficiently photoinduced, flowering begins at the apex and extends progressively downward along the stem (6). However, inhibition in the development of flower buds of *C. bipinnatus* was obtained by the use of long photoperiods after sufficient induction by short-day cycles (5). This retarding effect of subsequent treatment with long photoperiods was established later for *C. sulphureus* (2). Photoperiodic response is of a strictly localized nature in this species, since buds develop and flower only on the photoinduced portion or one node beyond (6).

The quantitative effect of the length of photoperiod manifested itself in data collected by AUSTIN (1). In plants treated with 8-hour light periods, buds appeared 11 days earlier than in plants on 12-hour photoperiod; after flower bud initiation there was no differential effect on floral development. Ten $7\frac{1}{2}$-hour photoperiods are sufficient to bring about the production of flowers and seeds in *Cosmos* (2). This reproductive phase is accompanied by a reduction in catalase activity (9) and by a marked accumulation of carbohydrate and protein in the stem tip (2). When fewer inductive photoperiods are followed by long-day cycles, the floral primordia tend to develop into "vegetative flowers" or interphases between normal flowers and vegetative shoots. If the induction is sufficiently limited, reversion to vegetative growth may occur. These anatomical changes toward foliaceous floral structures have been described by BIDDULPH (2).

Although flower buds appear on *Cosmos* exposed to twelve short light periods, STRUCKMEYER's work (17) indicated that eighteen short photoperiods are required to prevent the development of interphases or "vegetative flowers." Anatomical analysis of the stem showed that under photoinductive treatment cambial activity was decreased; when the plant was returned to an environment conducive to vegetative growth, meristematic activity was again resumed and was sometimes accompanied by the aborting of floral primordia.

Recent investigations (3) on *Cosmos* indicate that the "stimulus to flowering" produced during photoperiodic induction continues to pass from the leaves after they are returned to long photoperiods.

These anatomical and morphological changes in *Cosmos*, as responses to photoperiodic induction and postinduction treatment, suggest an attendant cytological upset with which this present study deals.

Material and methods

Seeds of *Cosmos sulphureus* Cav. var. Klondike (a short-day strain) were planted in flats on greenhouse benches on July 31, 1946. Immediately after germination the seedlings were placed on long photoperiods (8:00 A.M.–3:00 A.M.) obtained by supplementing the natural daylength with "Daylight" fluorescent lamps automatically controlled by clocks. When 2 weeks old, the seedlings were planted singly in 2-inch pots. On August 24 they were selected for uniformity of size and vigor and were transplanted in pairs into 4-inch pots.

On September 21, when all plants had at least five pairs of true leaves, twenty-three lots of nine pots each (eighteen plants) were subjected to differential light treatments (table 1). Six groups of

three lots each were placed on 8-hour photoperiods for 6, 7, 8, 10, 12, or 14 days, respectively. The three lots in each group received subsequent photoperiods of 14, 19, or 24 hours. Five lots of controls were employed. One lot was placed on natural daylength, another on 8 hours of light and 16 hours of darkness; both of these flowered profusely. The other controls on 14-, 19-, and 24-hour photoperiods did not flower. The photoinductive cycles of 8 hours of light and 16 hours of darkness were obtained by covering bench frames, built over the plants, with heavy black cloth at 4:00 P.M. and removing the cover at 8:00 A.M. the next morning. All plants were kept in two rooms of the same wing of the greenhouse in order to obtain environmental conditions that were as nearly identical as possible until the variable of light was introduced.

Floral buds were fixed in SAX's modification of Navashin's solution, washed in tap water, and run up in an ethyl-tertiary butyl alcohol dehydrating series

TABLE 1

SUMMARY OF TREATMENT AND HARVEST

Group no.	Placed on short photoperiod	Placed on long photoperiod	Total no. of photoinductive cycles	Postinduction treatment	Total no. of buds developed	First harvest	Last harvest
I	9/21/46	9/27/46	6	A. 14 hr.	2	11/18/46	.....
				C. 19 hr.	1	12/14/46	.....
				D. 24 hr.	1	1/14/47	.....
II	9/21/46	9/28/46	7	A. 14 hr.	14	11/ 8/46	1/22/47
				C. 19 hr.	8	11/ 7/46	1/ 2/47
				D. 24 hr.	8	11 /5/46	1/ 2/47
III	9/21/46	9/29/46	8	A. 14 hr.	22	11/ 5/46	1/12/47
				C. 19 hr.	19	11/ 7/46	1/12/47
				D. 24 hr.	15	10/30/46	1/ 2/47
IV	9/21/46	10/ 1/46	10	A. 14 hr.	25	11/ 4/46	1/ 2/47
				C. 19 hr.	28	10/31/46	1/12/47
				D. 24 hr.	17	11/ 4/46	1/12/47
V	9/21/46	10/ 3/46	12	A. 14 hr.	51	10/26/46	1/12/47
				C. 19 hr.	49	10/26/46	1/12/47
				D. 24 hr.	39	10/22/46	1/12/47
VI	9/21/46	10/ 5/46	14	A. 14 hr.	85	10/15/46	1/12/47
				C. 19 hr.	88	10/26/46	1/12/47
				D. 24 hr.	83	10/16/46	1/12/47

CONTROLS

Group no.	Placed on short photoperiod	Placed on long photoperiod	Photoperiod	First harvest	Last harvest
VII	9/21/46	.....	Natural	10/14/46	11/22/46
VIII	9/21/46	.....	8 hr.	10/10/46	11/11/46
IX	.....	9/21/46	14 hr.	.....	.....
XI	.....	9/21/46	24 hr.	.....	.....
XII	.....	9/21/46	19 hr.	.....	.....

into paraffin. Sections cut at 10μ were stained with Heidenhain's iron-alum haemotoxylin.

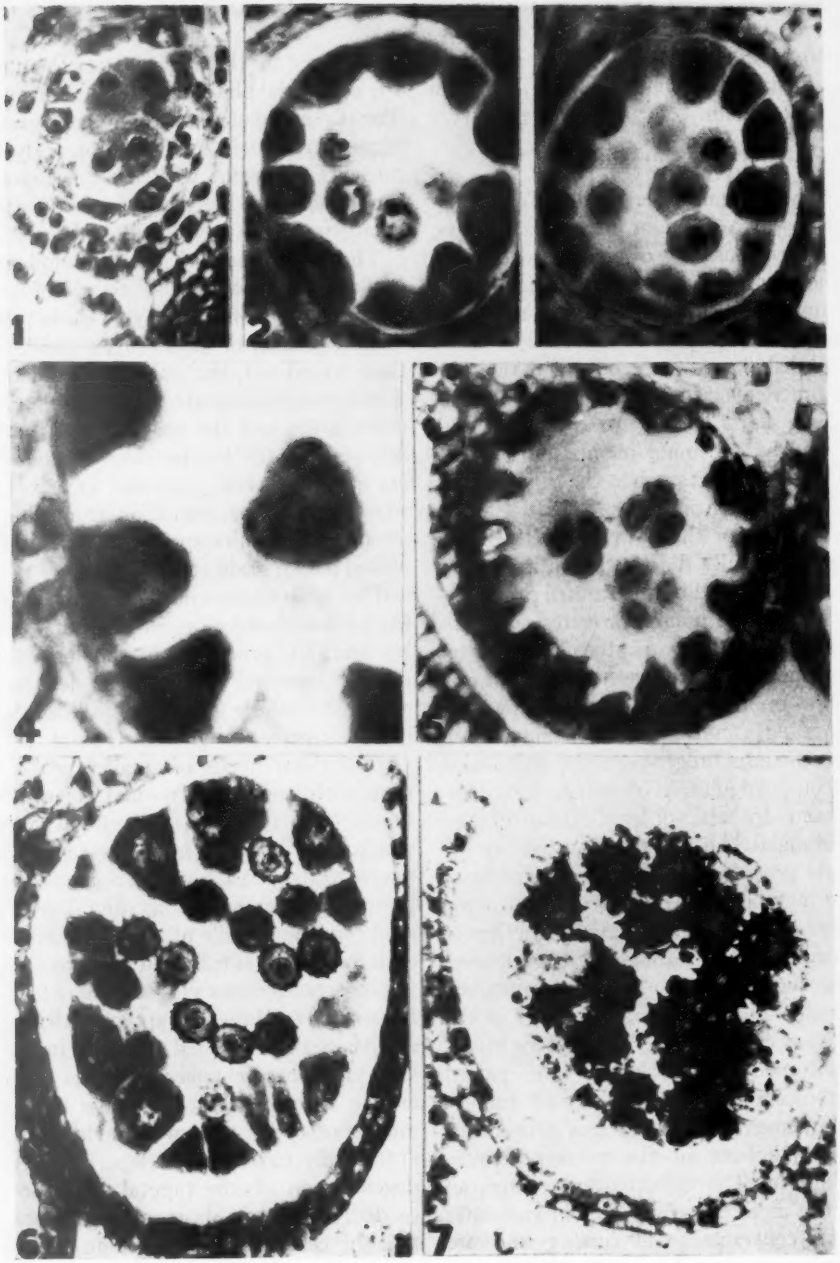
Observations

The normal flower head of *Cosmos* develops two rows of involucral bracts, consisting usually of eight in each row. The inner membranous bracts, surrounded by other foliaceous ones, bear the usual eight-ray flowers in their axils. The disk flowers are produced in the axils of bractlets on the determinate head. The young floral heads are completely inclosed by the inner membranous set of involucral bracts, while the outer bracts are spreading ones (fig. 31). In the development of the flower bud, the floral tube is usually differentiated first but is closely followed by the stamen primordia in which the microsporocytes soon differentiate. The sporocyte cells are larger and have slightly denser cytoplasm and more prominent nuclei than the surrounding somatic cells. Continued division of the sporogenous cells, which soon become angular in contour, is accompanied by the differentiation of the sporangial wall and tapetum (fig. 1).

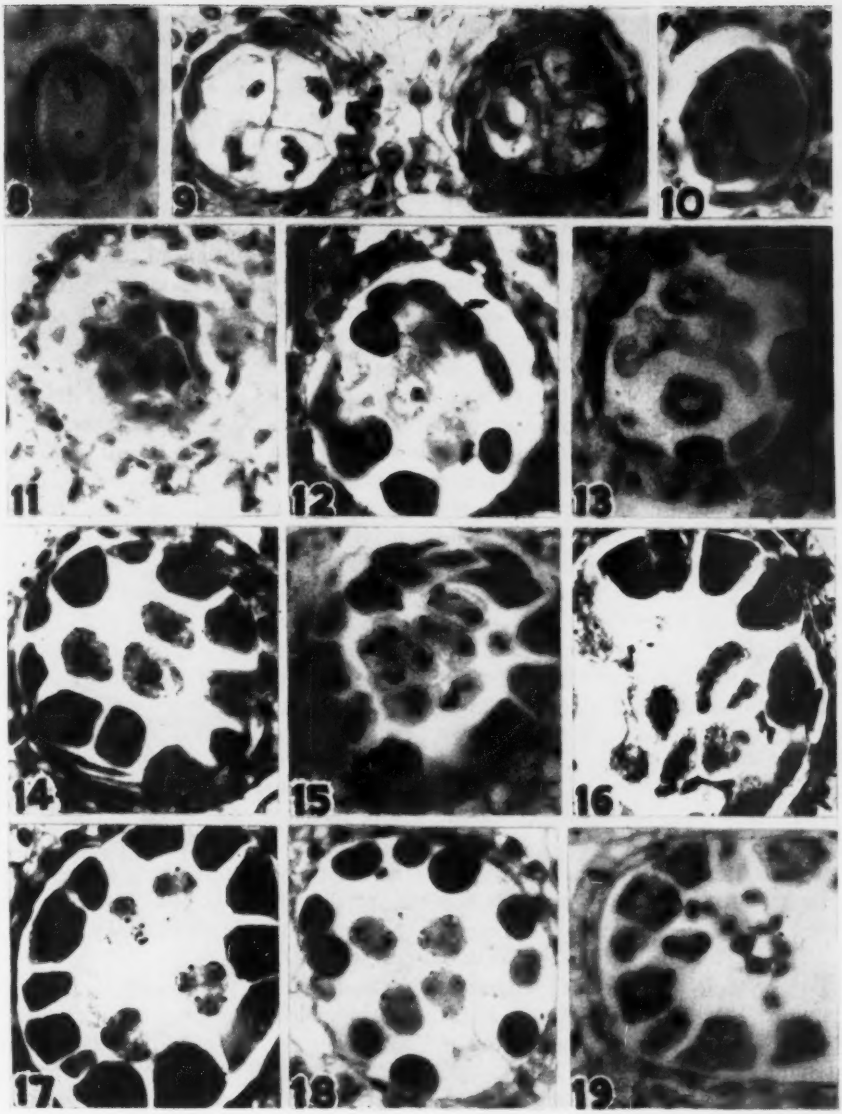
As growth proceeds, the reticulum of the metabolic nucleus gives way to the attenuated chromonemata of the leptotene, scattered throughout the nucleus. The pairing of the slightly contracted threads and an increased volume of the nucleus characterize the xygotene stage. Continued contraction of the paired chromosomes (pachytene) and further enlargement of the nucleus proceed as the cytoplasm of the microspoiocytes rounds up. The rounding-up is completed in the diplotene when the chromosomes have contracted still further and are evenly distributed near the nuclear membrane (fig. 2). Marked shortening of the chromosomes (diakinesis) is immediately followed by the alignment of them at the equatorial plate (metaphase I, fig. 3). The disjoining of the chromosomes (anaphase I) results in the formation of dyad nuclei. Concurrent with the developing prophase stages are the division and the enlargement of tapetal cells.

The second meiotic division follows immediately, giving rise to the four-nucleate stage (fig. 4). Cytokinesis produces a quartet of spores (fig. 5), and, as these round out, the walls of the microsporocytes disintegrate. The tapetal cells disintegrate and the residue from them diffuses into the locular cavity, producing a translucent, granular matrix in which occurs the transition from thin, smooth-walled microspore to thick, spiny-walled pollen grain (figs. 6, 7).

The individuals most responsive to the photoinductive cycles in groups IV, V, and VI produced morphologically normal terminal flowers. The less responsive in these groups, as well as the most sensitive in groups II and III, yielded floral heads of smaller proportions with longer than normal foliaceous bracts (figs. 32, 33, 34). The floral buds which were initiated later developed at varying rates in each group. Some of the primordia remained in an undifferentiated condition, while others early differentiated the floral tube and stamens containing sporogenous cells and tapetum. In groups II–VI the first apparent degenerative changes seemed to occur in the tapetum. These changes began as early as the metabolic nuclear stage of the microsporocytes and were evident as abnormally narrow cells (figs. 8, 9). As degeneration of the tapetal cells proceeded, their cytoplasm stained black, and the cells sometimes became oval in shape (fig. 12). More frequently, however, they remained narrow (figs. 8, 22, 23) or became angular (figs. 13, 14) in



FIGS. 1–7.—From control plants of group VIII. Fig. 1, young microsporocytes, normal tapetum, and sporangial wall. Fig. 2, diplotene. Fig. 3, metaphase. Fig. 4, four-nucleate stage. Fig. 5, quartet of microspores. Fig. 6, microspores. Fig. 7, pollen grains.



FIGS. 8-19.—Fig. 8, microsporocytes surrounded by degenerating tapetal cells, lot IV-D. Fig. 9, microsporocytes with little or no cytoplasm, surrounded by darkening tapetum, lot IV-A. Fig. 10, sporogenous tissue darkening, tapetum narrow and degenerating, lot II-D. Fig. 11, one locule with degenerating microsporocytes and outer sporangial wall containing heavily staining globular bodies, lot III-D. Fig. 12, locule of anther with globular tapetal cells and disintegrating sporocytes, lot IV-C. Fig. 13, locule containing narrow and angular tapetal cells inclosing normal microsporocyte and large denucleated plasmodial mass, lot IV-A. Fig. 14, locule containing angular degenerating tapetal cells with heavy black granules floating in cytoplasm, lot IV-D. Fig. 15, degenerating tapetum and lagging chromosomes in meiosis I, lot II-A. Fig. 16, degenerating tapetum and disintegrating dyads with extra-nuclear chromosomes, lot II-A. Fig. 17, degenerating tapetum and tetrads with extra-nuclear chromosomes, lot IV-D. Fig. 18, rounded, disintegrating tapetal cells and tetrads with extra-nuclear chromosomes, lot IV-A. Fig. 19, incipient stages of tapetal degeneration and advanced degeneration of dyads and tetrads, lot II-C.

outline. Less often observed were the darkly stained microsporocytes and vacuolated cells of the tapetum as shown in figure 10.

Degenerating cytoplasm in microsporocytes was of common occurrence. In a few cases heavily staining globular bodies in the outer sporangial wall accompanied these deteriorating microsporocytes (fig. 11). Coincident with these responses of the tapetum were two degenerative reactions of the sporogenous cells. In the extremely suppressed floral buds of groups I–VI the prophases seemed not to be initiated; the cytoplasm of these microsporocytes became highly vacuolate with the nucleus markedly contracted. Such cells appeared to develop no further (figs. 9, 29). On the other hand, in less suppressed buds of groups II–VI the cytoplasm of the sporogenous cells sometimes appeared normal and prophases might continue regularly; frequently some of the sister sporocytes developed into large plasmodial masses which were two to four times the average size of normal microsporocytes (fig. 13).

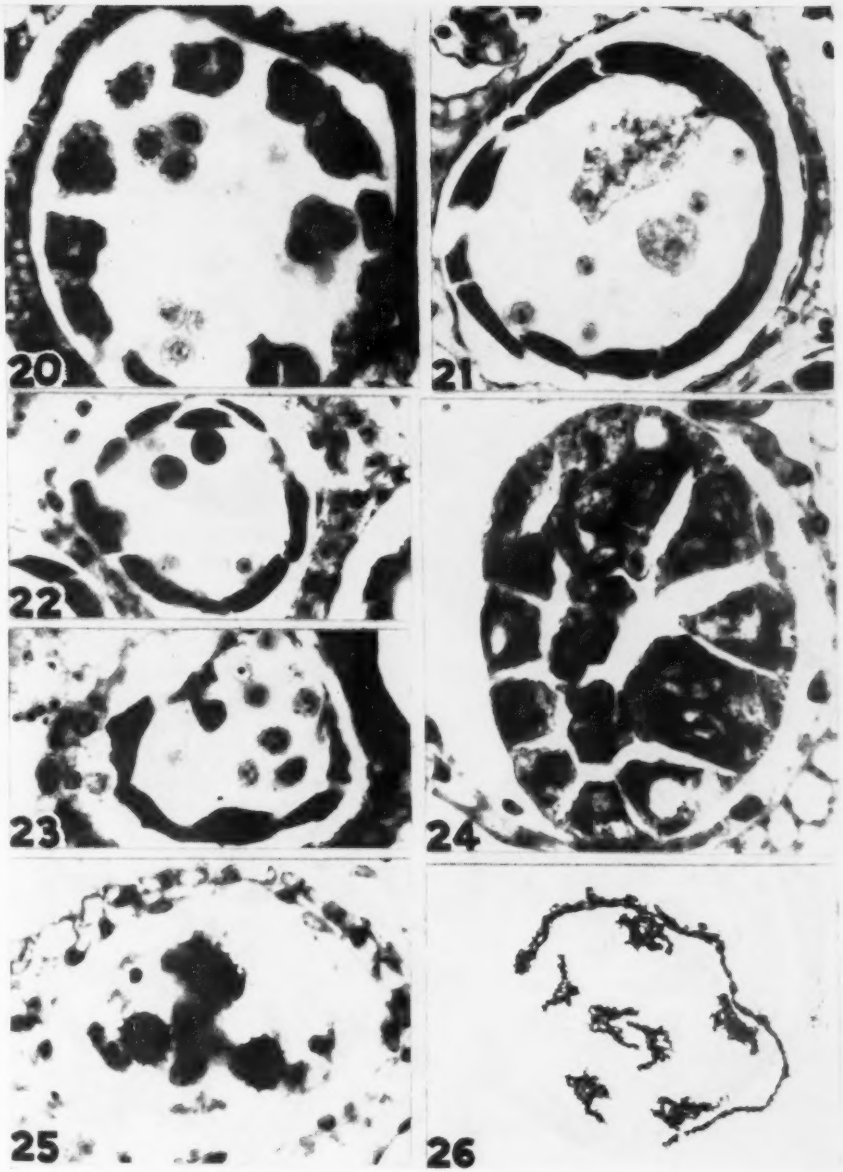
At the time of late diakinesis and metaphase I, a condition frequently observed in the microsporocytes in groups II–VI was the occurrence of darkly staining cytoplasm with scattered, heavily stained, black granules (fig. 14). A less frequent abnormality was the lagging of chromosomes in anaphase I (fig. 15). These chromosomes could still be seen after the second meiotic division was completed (fig. 17). This occurrence was also noted in association with rounded tapetal cells (fig. 18). Where flower suppression was greater, degeneration often took place at the two-nucleate stages and chromosomes could be seen floating in the dark-stained cytoplasm near the partially reconstituted nuclei. Shapeless masses of both heavily stained and highly vacuolate protoplasm also occurred (fig. 16).

Although degeneration of sporogenous tissue at any stage of development was usually preceded or accompanied by marked deterioration of tapetal cells, occasionally a severe upset in meiosis occurred, although tapetal cells showed only the incipient stages of cytoplasmic degeneration (fig. 19).

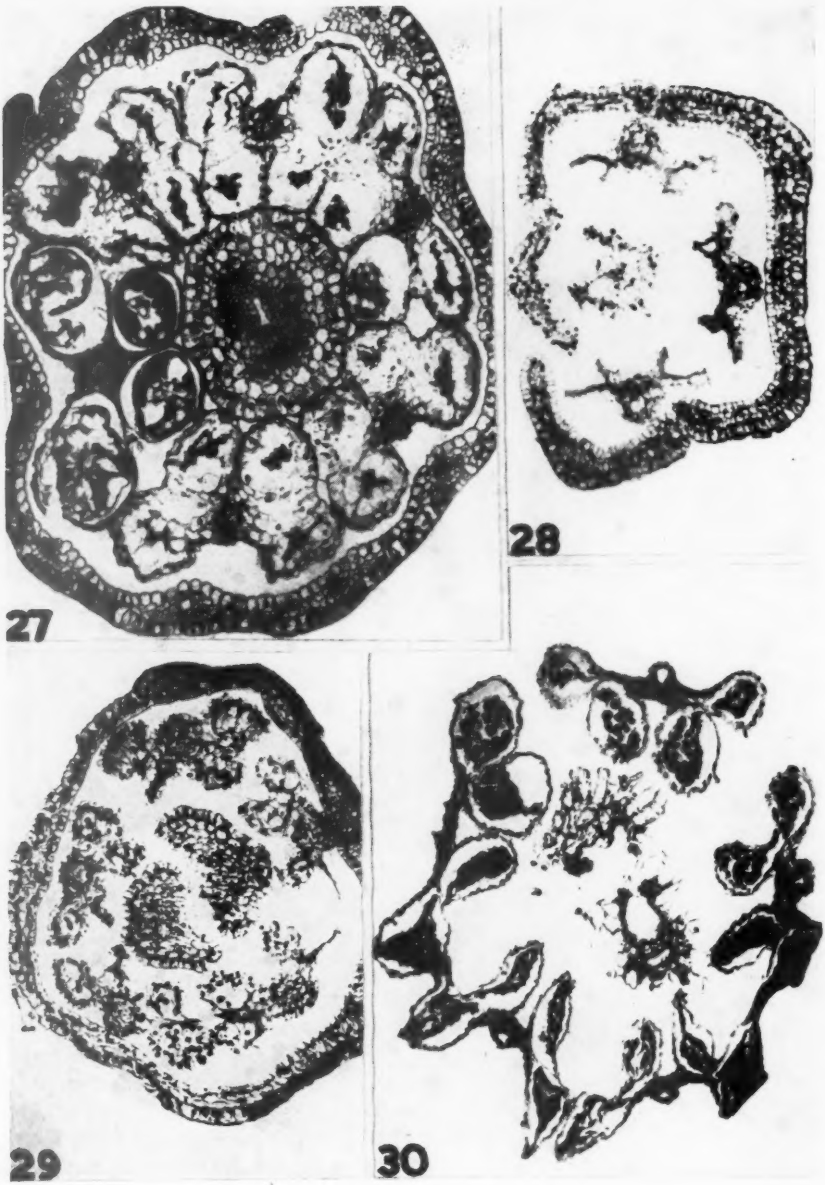
In the plants in groups II–VI in which buds developed to a fuller extent than did those just mentioned, and lagging of chromosomes was not apparent, cytoplasmic changes first appeared immediately exterior to the intensely stained nucleus (fig. 20). Position and procedure of degeneration were similar in both tetrads and microspores (fig. 22). Of less frequent incidence, apparently normal microspores occurred in conjunction with irregular masses of granular cytoplasm that showed evidence of almost complete nuclear impairment (fig. 21). When degeneration of tapetal cells was as marked as is shown in figures 21, 22, and 23, many microspores did not develop further. With less deterioration in the tapetum, the microspore wall began to thicken and the spores became angular in appearance (fig. 24).

Groups II–VI produced a few flowers which yielded some morphologically normal pollen. However, in the locules containing an abnormally dark-staining matrix small, shrunken, black-stained bodies (deteriorated microspores) appeared along with apparently normal pollen grains (fig. 25).

Groups I–VI also showed more pronounced degeneration than just described, in which the locules were filled with an indistinguishable, deteriorated mass of sporogenous tissue. The terminal flower bud from lot II–C, having seven



FIGS. 20–26.—Fig. 20, locule with deteriorating tapetum surrounding quartets of spores which show cytoplasmic degeneration immediately exterior to nuclear membrane, lot V-D. Fig. 21, locule with narrow, blackened tapetal cells surrounding a few normal microspores and two plasmodial masses with evidences of deteriorated nuclear material, lot III-A. Fig. 22, locule of anther with narrow, degenerating tapetal cells and degenerating microspores, lot II-C. Fig. 23, locule containing darkening sporangial wall, degenerating tapetum, and deteriorating microspores, lot IV-A. Fig. 24, locule with abnormally darkened tapetal cytoplasm filled with large granules and disintegrating angular microspores, lot II-D. Fig. 25, locule containing several morphologically normal pollen grains, many shrunken grains, and heavily staining tapetal matrix, III-A. Fig. 26, flower from lot II-A with shriveled anthers and floral tube.



FIGS. 27–30.—Fig. 27, one flower from floral head in fig. 33; stages of tapetal and sporogenous degeneration, lot II-C. Fig. 28, flower from floral head in fig. 34 with three stamens of shriveled anthers and floral tube, lot III-D. Figs. 29, 30, flowers from two first-node (below terminal) buds of same plant, lot V-D; fig. 29, flower having locules with vacuolated sporogenous tissue; fig. 30, flower from floral head in fig. 32 with partially collapsed locules containing abnormal pollen.

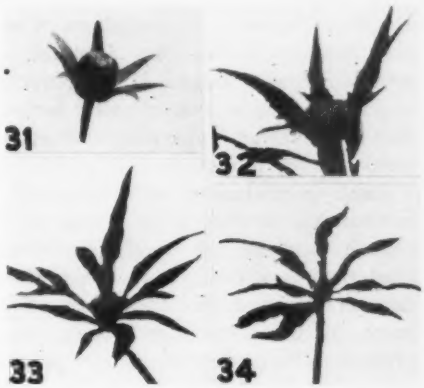
photoinductive cycles followed by a 19-hour photoperiod, illustrates this condition. This head with longer than normal foliaceous bracts and inhibited membranous ones (fig. 33) produced disk flowers of the type pictured in figure 27. Notable is the fact that the locules shown are in different stages of development and deterioration. The most advanced type of deterioration shows mere rudiments of shriveled anther walls, filaments, and floral tubes (fig. 26). Not uncommon were the abnormal numbers of three and four stamens instead of five per flower. A terminal bud (fig. 34) in lot III-D, having eight photoinductive cycles followed by continuous light, produced a number of three-stamen flowers (fig. 28). For the most part in these entirely suppressed heads all the flowers were in the same stages of degeneration. However, in instances of less retardation, differences in stage of development of flowers within one head led to a range from abnormalities in early microsporogenous tissue to small, collapsed pollen grains.

The gross floral response of a first-node (below the terminal) flower to twelve photoinductive treatments, followed by continuous light, is shown in figure 32. The foliaceous bracts were 10–15 mm. in length; the membranous ones were completely inhibited, resulting in a head of unprotected, widely separated flowers. Cytological examination revealed that this bud, the larger of two first-node buds, had produced abnormal pollen (fig. 30), while the smaller one had been inhibited in its development before the initiation of the prophases (fig. 29). These early sporogenous stages showed cells from which the cytoplasm had completely disappeared.

In these experiments a few buds were produced in the six-cycle material in contrast to the results obtained by other workers (**2, 3**). Two of these buds showed collapsed sporangial walls containing degenerate pollen, and the others were not developed beyond the early differentiation of the floral tube.

Discussion

The effects of an insufficient number of short-photoperiod cycles in *C. sulphureus* Cav. var. Klondike are similar in many respects to those described by NIELSEN (**11**) in Biloxi soybeans having five to ten photoinductive cycles followed by a long photoperiod. His results showed that sporogenous tissue failed to develop further than the early prophases of meiosis in plants given five or fewer short photoperiods. Under this condition the sporocytes enlarged, became vacuolated, eventually lost their nuclei, and disintegrated. Corresponding consequences were found in carbohydrate-deficient tomatoes, as reported by HOWLETT (**8**). Severe carbohydrate deficiency



FIGS. 31–34.—Fig. 31, terminal bud from control group VIII. Fig. 32, first-node bud from lot V-D. Fig. 33, terminal bud with long foliaceous outer involucral bracts and inhibited inner one from lot II-C. Fig. 34, terminal bud with abnormally long foliaceous outer bracts and partially inhibited inner ones from lot III-D.

produced a retarding effect on the development of the flower with resultant collapse of cell walls and the degeneration of sporogenous cells before initiation of the prophases. The degree of degeneration was correlated with the severity of the deficiency.

Soybean plants given six to ten short photoperiods frequently showed degeneration at later stages in meiosis than did the five-cycle groups. Buds from these plants showed deterioration after the first division, such as failure of reconstitution of the four chromosome groups following meiosis II, or frequent degeneration of microspores after formation in the tetrad. Some plants receiving eight or more inductive cycles produced a small percentage of morphologically normal microspores. It was concluded that in soybean the number of short photoperiods influences the degree of degeneration and the stage of development to which microsporocytes may progress. The long postinductive photoperiod appeared to effect further the suppression of flowers and the degenerative changes observed (11).

In the markedly suppressed buds of *Cosmos*, no differentiation of flower parts and sporogenous tissue occurs, although grossly a rounded protuberance can be recognized. A somewhat less retarded floral structure shows differentiation of the floral tube, anthers with well-developed sporangial wall, threadlike black tapetum, and sporogenous cells with little or no cytoplasm (fig. 29). This condition in sporogenous tissue concurs with data on tomato (8) and soybean (11) in which development of the sporogenous cells fails to reach the prophase stages of meiosis.

In cases in which the prophases were initiated in the sporogenous tissue in tomato, lagging chromosomes in meiosis II were reported. Likewise in *Cosmos*, similar lagging was noted in both meiotic divisions in groups II, IV, and V and was always accompanied by a heavily stained tapetum. Failure to proceed beyond meiosis I was observed in many cases (fig. 16). On the other hand, if reconstruction of tetrad nuclei did occur, chromosomes were often found in the cytoplasm outside of the nuclear membrane (figs. 17, 18).

Cells with fully reconstituted nuclei may develop further, but degeneration of microspores often occurred directly following cytokinesis, as in soybean, or later before the microspore wall thickened. Growth of the microspore wall and production of spiny protuberances of the pollen-grain wall are apparently subordinate to the presence of at least some matrix formed from the contents of the tapetal cells. Unless floral development is suppressed sufficiently to prevent initiation of the prophases in all flowers, morphologically normal pollen may be produced in a large percentage of flowers along with numerous degenerative developmental stages.

Since the flowers of *Cosmos* are produced in a progressive fashion from apex downward along the stem (6), comparisons of all terminals, or all first-node flowers, or all second node flowers below the terminal, were made of the experimental material. However, in so far as all the plants are not equally sensitive (2) and the stimulus to flowering is inactivated by young expanding leaves present during the induction period (3), a wide range of variation was encountered microscopically. This was true, not only within an experimental lot, but within buds of the same node on one plant (figs. 29, 30) as well as within the same flower (fig. 27). This considerable overlapping makes it impossible to draw lines

of demarcation cytologically, between the differentially treated lots, even though a quantitative record of buds of the experimental lots expresses a retardation in total reproductive activity in correspondence with reduced photoinduction. An inhibitory effect of the long postinductive cycles on total flower bud production is suggested in the column of bud numbers in table 1. These results are in accord with those obtained in *Cosmos* by BIDDULPH (2) and in soybean by NIELSEN (11).

When *C. sulphureus* plants are treated with a sufficient number of short-photoperiod cycles, a series of chemical reactions appears to be initiated. If these plants are subsequently placed under favorable light and other environmental conditions, they proceed to flower normally. The chain of chemical reactions may be upset at various points if the plants are given an insufficient number of short photoinductive periods or subsequent long photoinductive treatments of 19 or 24 hours of light. This indicates that a complex interacting system, perhaps hormonal in nature, is operative. Disruption at any place in such a chain of reactions as a consequence of the influence of an unfavorable photoperiod causes a variety of morphological and cytological abnormalities.

Summary

1. Experimental groups of *Cosmos sulphureus* Cav. var. Klondike were given photoinductive cycles of 8 hours of light and 16 hours of darkness for 6, 7, 8, 10, 12, and 14 days. The groups were then divided into three lots each, which were given differential postinductive photoperiods of 14, 19, or 24 hours.

2. Controls were run on natural and 8-hour photoperiods; both of these lots flowered profusely. Three other controls run continuously on 14-, 19-, and 24-hour photoperiods did not flower.

3. Flower buds were collected and fixed successively from all groups. These provided a normal series for floral and meiotic development through pollen formation and material for the study of abnormalities induced by the experimental photoperiods.

4. The markedly suppressed buds of *Cosmos*, usually surrounded by long foliaceous outer bracts and inner inhibited ones, showed no differentiation of floral parts or sporogenous tissue. Less retarded reproductive structures produced floral tube, sporangial wall, a black-line tapetum, and sporogenous cells with little or no cytoplasm. These sporocytes did not initiate the prophases.

5. In plants in which retardation was less marked, various abnormalities occurred, such as degeneration of tapetum, plasmodial masses derived from sporogenous tissues accompanying the normal stages, lagging of chromosomes and the failure of some of them to be included in the microspores, and degenerating cytoplasm in the microspores.

6. No pollen which appeared to be morphologically normal occurred in the anthers of flowers in which tapetal cells did not form a locular matrix.

7. Although a quantitative record of buds of the experimental lots clearly shows a reduction in total reproductive activity in relation to reduced photoinduction and to increased length of the postinductive photoperiod, it is cytologically impossible to draw lines of demarcation between the lots.

The writer wishes to express appreciation to Dr. J. M. BEAL for guidance and suggestions throughout the course of this investigation.

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PHOTOPERIODIC RESPONSES OF GEOGRAPHICAL STRAINS OF ANDROPOGON SCOPARIUS[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 591

EUGENE C. LARSEN

Introduction

Andropogon scoparius Michx. was chosen for investigation because of its extremely wide distribution and recognized diversity (**6, 7, 10, 12, 16, 22**) and because of its importance as a forage grass in many parts of the United States. It has recently been used in the reseeding of abandoned, cultivated land, especially in the central prairie region (**8, 9, 11, 15, 21**). It is also quite well adjusted to growth in greenhouses during the hot summer months in Chicago.

Information on the photoperiodic re-

[1] A report of an investigation carried on at the University of Chicago at the suggestion and under the supervision of Dr. CHARLES E. OLMSTED as one in the series "Growth and development in range grasses." This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

sponses of *A. scoparius* should be of value in analyzing the noticeable variations among the ecotypes when they are grown together under greenhouse or nursery conditions (10) and in understanding and explaining the reproductive and vegetative habits of the species. Such information may be of use in varietal selection and in the interpretation of growth and flowering habits of strains grown outside their native habitats. It should also be of interest to those studying the evolution and natural selection of adaptive physiological responses and their relation to plant migration.

There has been relatively little work on photoperiodic responses of various strains of native forage and range plants, although the literature in the general field of photoperiodism is extensive. The latter has recently been summarized by HAMNER (13), WHYTE (23), and BORTHWICK (5). OLMSTED (19) has referred to general literature related to photoperiodic work on grasses.

A. scoparius is surpassed by few species of native American plants in its wide range of habitat and distribution. It has, according to HITCHCOCK (14), been collected in all the states except Washington, Oregon, Nevada, and California. From the standpoint of abundance, its center of distribution is in the tall-grass prairie region between Texas and the Canadian line. Here it was one of the species which made up the major part of the native vegetation of the original prairie.

Its value as a forage plant varies considerably, both with season and with locality. It is generally palatable from the time it starts to grow in the spring until it begins to mature. ROGLER (20) listed *A. furcatus* as the most palatable of all the important warm-season grasses of the prairie states and stated that *A. scoparius* was its equal until around August 10, when the culms began to mature. *A. scoparius* was found to be as palatable as any grass on the range in North Dakota during the period from July 12 to 18. It does not withstand heavy spring grazing.

The range of morphological variation in this species complex has led to the recognition of several intergrading forms and varieties (12, 14, 16). HUBBARD (16) stated that the variations may be in color of the plant, length of the sessile spikelet, villousness of the sheath and leaves, compression of the sheath, or length of the hairs at the internodes of the rachis. Some of the described varieties and specific segregates have fairly well-marked geographical ranges.

Specimens of the twelve collections used in this study were sent to the National Herbarium in Washington, D.C., for identification. Dr. J. R. SWALLEN, associate curator, pointed out that strain 2 differs slightly from the others in that it has a broad, more sharply keeled lower sheath which is villous and that the staminate or pedicellate spikelets are about as large as the sessile, fertile ones. He thought that this strain is probably *A. divergens*, a species closely allied to *A. scoparius* and sometimes listed as a variety of it. He considered the eleven other collections to be *A. scoparius* and, although recognizing variation among them, did not assign varietal rank.

No cytological observations have been made upon the plants used in the present experiment. Cytotaxonomic studies (6, 7, 17) of the species and some of its named varieties have shown most of the investigated individuals to be tetraploid plants ($2n = 40$), although differences in structural details of the chromosome complements are correlated with some of the observed morphological variations.

Several workers have contributed to

our recent knowledge of physiological variation exhibited by various ecotypes in grasses. NIELSEN (18), for *Panicum virgatum*, has shown what a complex of diversity one is likely to find when dealing with a large number of ecotypes from widely separated areas. OLMSTED (19), for side-oats grama, found that practically the entire range of reported photoperiodic responses was exhibited by the different ecotypes. He stated that this species cannot be classified photoperiodically except with reference to strains.

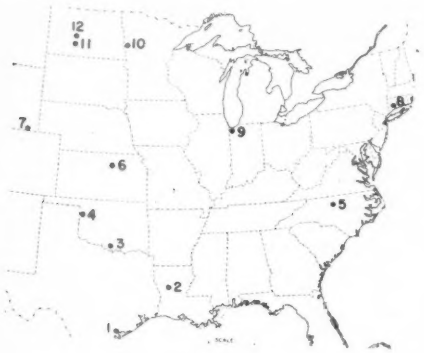

FIG. 1.—Geographical sources of strains numbered as shown.

CORNELIUS (10) has recently described the effects of source of seed of *A. scoparius* on growth, adaptation, and its use in revegetation. In his work at Manhattan, Kansas, with sixteen strains of *A. scoparius* collected from widely separated geographical areas, he reported a difference of 126 days between the first flowering dates of the northern and southern ecotypes. His northern types were earlier in maturity and lower in forage production than the southern ones. The plants originating in central latitudes were intermediate. He obtained a high seed set in the latter plants. The northern ones suffered from hot winds, and the southern ones from frost injury. He found a highly significant correlation between the flowering date and the dry weight of a plant at the close of the season—early flowering led to low total yield. Winter injury was observed among his southern ecotypes.

The present experiment on photoperiodic responses of geographical strains of *A. scoparius* was initiated in the spring of 1946 and continued through the spring of 1947 in the greenhouses and experimental garden at the University of Chicago.

Material and methods

Forty plants, representing twelve points of origin (fig. 1) were assembled at the University of Chicago in March and April, 1946, through the courtesy of the collectors listed in table 1, and were assigned the indicated designations. Each plant was assumed to be, and apparently was, an individual clone.

On May 1 each of the forty clones was divided, and the divisions, after being trimmed to approximately equal size, were planted in 8-inch, unglazed clay pots. Three segments of a clone were planted in each of five pots to make five identical series of forty pots each.

The pots were filled with soil consisting of a mixture of six parts of coarse sand with five parts of black loam garden soil. No fertilizer was added. This light, sandy-textured soil was used to emulate as nearly as possible the soils found in the native habitats of many of the plants.

One series (*N*) was placed on a greenhouse bench, and another (*G*), after being potted for a 10-day period, was transplanted to an experimental garden. Series *N* and *G* received Chicago natural daylength (interval between sunrise and sunset on June 21 is 15 hours and 14 minutes). The other three series were placed on movable trucks which were

wheeled into separate lightproof, ventilated sheds at 5:00 P.M. and out again at 8:00 A.M. They thus received 9 hours of natural light per day. Supplementary illumination to extend their light periods was supplied by means of three 200-watt incandescent-filament lamps mounted in individual reflectors over each of the trucks. These lights, controlled automatically, were turned on at 5:00 P.M. and were shut off at the hour necessary to provide total consecutive photoperiods of 13, 14, and 15 hours for the three series. Intensity of supplementary illumination at average foliage height ranged from 100 to 180 foot-candles, depending on the height of a plant and its position on the truck.

Each series was so arranged that the taller plants of southern origin did not shade the smaller northern ones. As they developed, many of the taller culms were staked and tied to prevent them from falling over, breaking off, or shading surrounding plants. Pots were watered adequately to prevent temporary wilting even during hot sunny weather. Sow bugs and insect pests were controlled by the usual methods, and there were no fungus diseases of importance.

TABLE 1

SOURCE OF TWELVE STRAINS OF ANDROPOGON SCOPARIUS

Strain and clone	Point of origin of clone or parental seed lot	Approximate latitude	Clone secured from	Sent by	Accession number of agency
12*A*	Price, N.D.	47° 10′	Northern Great Plains Field Station, Mandan, N.D.	George A. Rogler, BPI, Mandan, N.D.	D-139-3
11*B*, *C*	Mandan, N.D.	46° 50′	Northern Great Plains Field Station, Mandan, N.D.	George A. Rogler, BPI, Mandan, N.D.	D-138-3 (B) D-137-4 (C)
10*A*, *B*, *C*, *D*, *E*	Moorhead, Minn.	47° 00′	Field	J. J. Westfall, Moorhead, Minn.	
9*A*, *B*, *C*, *D*, *E*	Dunes State Park, Ind.	41° 30′	Field	E. C. Larsen	
8*A*, *B*, *C*, *D*	North Haven, Conn.	41° 23′	Field	H. J. Lutz, Yale University, New Haven, Conn.	
7*B*	Cheyenne, Wyo.	41° 05′	Northern Great Plains Field Station, Mandan, N.D.	George A. Rogler, BPI, Mandan, N.D.	D-6-11
6*B*, *C*	Manhattan, Kan.	39° 10′	SCS Nursery, Manhattan, Kan.	M. D. Atkins, SCS, Manhattan, Kan.	KG-1580*(B) KG-480 (C)
5*A*, *B*, *C*, *D*, *E*	Durham, N.C.	36° 00′	Field, Duke Forest	H. J. Oosting, Duke University, Durham, N.C.	
4*A*, *B*, *C*, *D*	Arnett, Okla.	36° 10′	Field	James E. Smith, Jr., SCS, Woodward, Okla.	
3*A*	Ardmore, Okla.	34° 10′	SCS Nursery, Manhattan, Kan.	M. D. Atkins, SCS, Manhattan, Kan.	KG-753
2*A*, *B*, *C*, *D*, *E*	Alexandria, La.	31° 15′	Field	J. T. Cassady, U.S. Forest Service, Alexandria, La.	
1*A*, *B*, *C*, *D*, *E*	Matagorda Bay, Jackson County, Texas	28° 15′	SCS Nursery, Tuscon, Ariz.	L. P. Hamilton, SCS, Tuscon, Ariz.	A-2966†

* Improved selection from KG-480.

† Grown from seed from San Antonio, Texas, collection SA-4437.

Observations on culm elongation and flowering were made twice a week or oftener. Heights of the three longest tillers (to tip of outstretched leaf or inflorescence) on each plant were measured on the first and fifteenth day of each month. The three values were averaged.

On November 1, 1946, all plants were harvested by clipping just above the soil line. Data were obtained on the total numbers of elongated and unelongated tillers. The central culm of each plant was analyzed for total length and for numbers of visible leaves, nodes, elongated internodes, and flowering branches. If inflorescences were not visible, the growing points were dissected to show whether flowers or flower primordia were present. After these measurements had been taken, dry weights of tops were obtained by the usual methods.

The *N* series was then placed back on the greenhouse benches, where most of the plants remained dormant during the winter months in spite of the warm temperatures. The plants in the garden (series *G*), after clipping on November 1, were left there to test their survival over winter at Chicago. Strains 1, 2, and 5 winter-killed in the garden. All other plants survived out-of-doors.

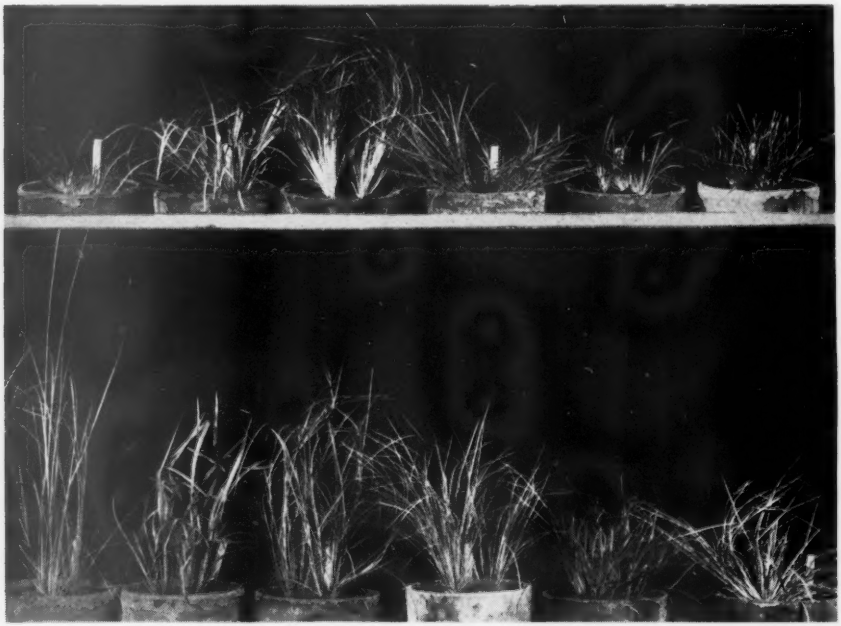

FIG. 2.—Twelve strains of *A. scoparius* grown on photoperiods of 13 hours. *Left to right, below*, strains 1, 2, 3, 4, 5, and 6; *above*, strains 7, 8, 9, 10, 11, and 12. This photoperiod was not long enough to induce internodal elongation. Southern strains show greatest leaf elongation. (Photographed September 1, 1946.)

Results

FLOWERING RESPONSES

The date of first flowering of a strain in any series was recorded as the date on which anthers were first exserted (first anthesis) by the first clone to flower. The

date of last flowering of a strain is defined as that on which the last-formed inflorescence in its clonal population came into anthesis. The length of flowering season of a strain was measured as the interval between these two extreme dates.

None of the twelve strains flowered on 13-hour photoperiod (table 2, fig. 2). Plants of only strains 2, 3, 4, and 5 produced visible inflorescences on the 14-hour light period (fig. 3), although on November 1, at the time of harvest, well-developed floral primordia were present in strain 1. Strains 6–12 neither flowered nor showed elongation on 14-hour photoperiod. In the 15-hour series, strains 4 and 6–12 produced flowers (fig. 4), but

FIG. 3.—Twelve strains of *A. scoparius* grown on photoperiods of 14 hours. *Left to right, below*, strains 1, 2, 3, 4, 5, and 6; *above*, strains 7, 8, 9, 10, 11, and 12. Southern strains 1–5 showed culm elongation and flowering. Strains 6–12 remained vegetative. Strain 9 from sand dunes of Lake Michigan showed greatest vegetative growth among central and northern strains. (Photographed September 1, 1946.)

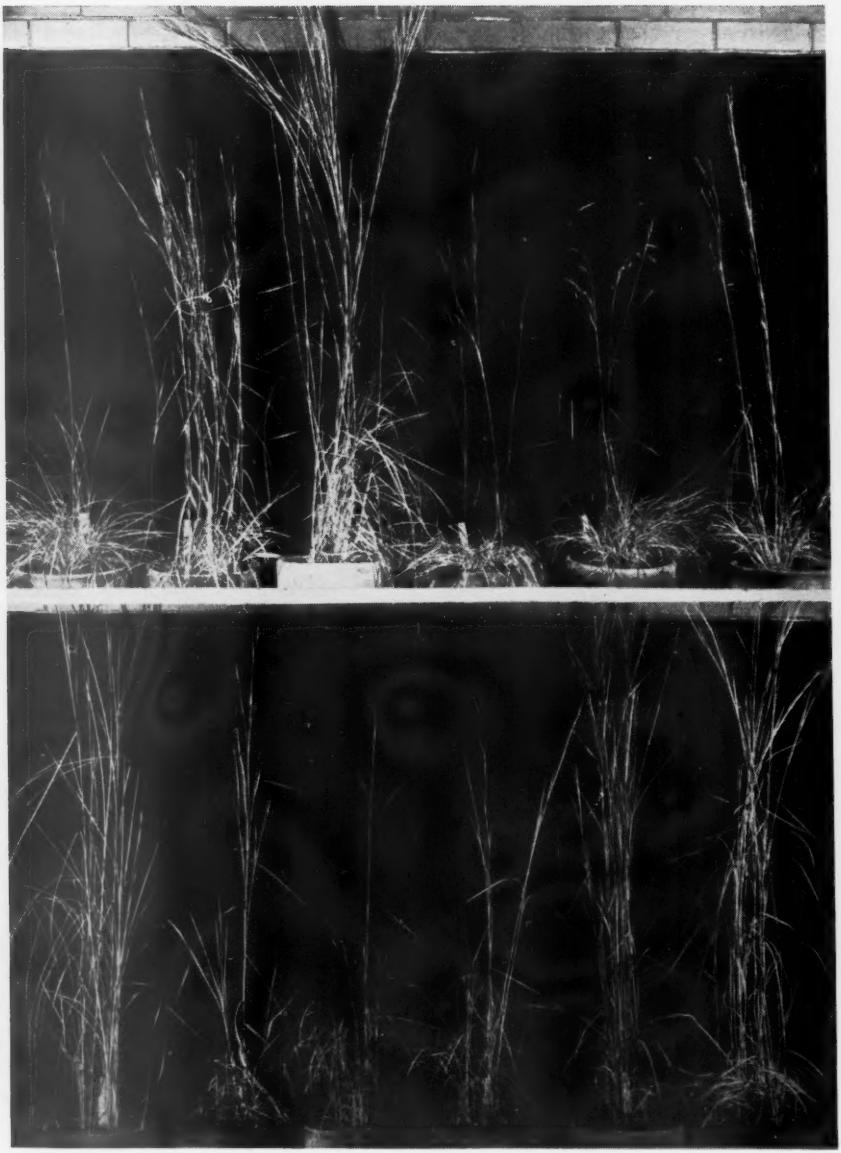

FIG. 4.—Twelve strains of *A. scoparius* grown on photoperiods of 15 hours. *Left to right, below*, strains 1, 2, 3, 4, 5, and 6; *above*, strains 7, 8, 9, 10, 11, and 12. All strains with elongated culms. Strains 1, 2, 3, and 5 did not produce flowers by time of harvesting. All plants were still green on harvesting date, November 1. (Photographed September 1, 1946.)

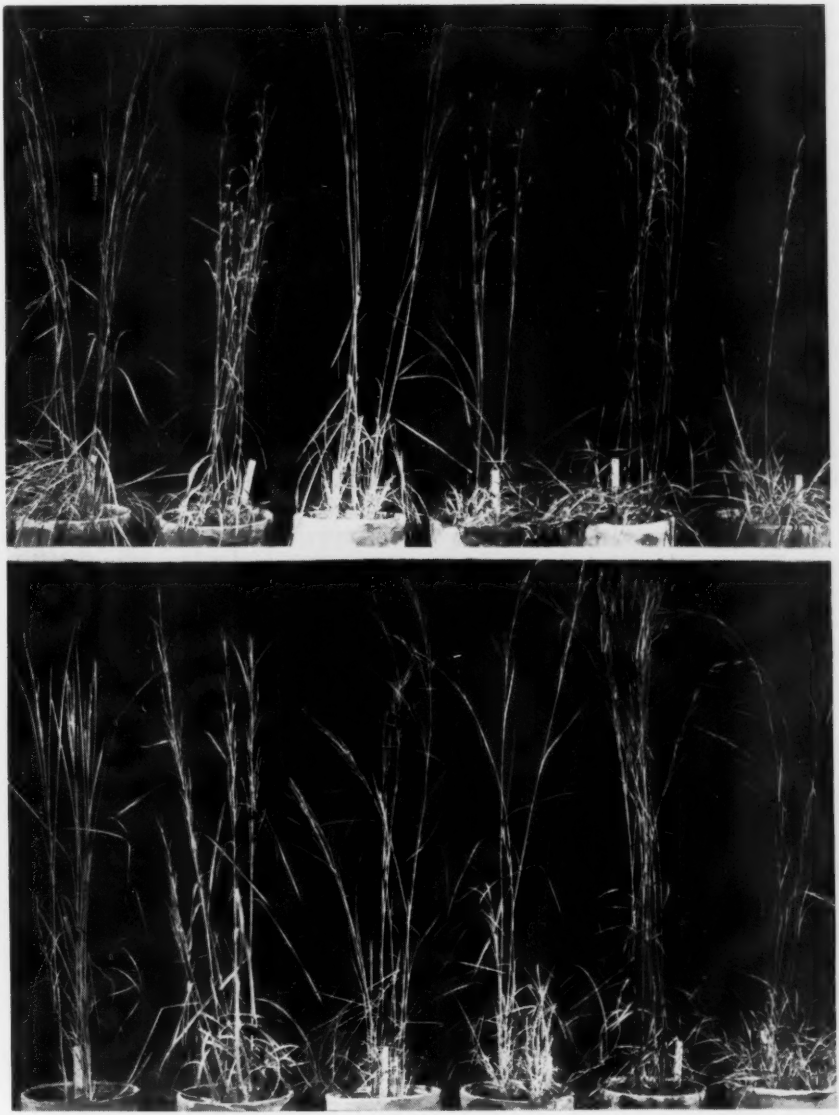

FIG 5.—Twelve strains of *A. scoparius* grown on Chicago natural daylength in the greenhouse (*N* series). *Left to right, below*, strains 1, 2, 3, 4, 5, and 6; *above;* strains 7, 8, 9, 10, 11, and 12. All strains flowered except strain 6. Northern strains have begun to enter dormancy. (Photographed September 1, 1946.)

the southern strains 1, 2, 3, and 5 had not visibly flowered at harvest time, even though culms were elongated. Dissection of their growing points showed that three of fifteen plants in strain 2, and five of ten in strain 5, had poorly developed floral primordia. Representatives of all strains flowered in the *N* series (fig. 5) except strain 6, which showed a low percentage of elongated culms. In the *G* series all clones flowered except 2*C*, 2*E*, 6*C*, and 10*D*. Plants 2*E* and 10*D* did not flower in any series.

TIME TO FIRST FLOWERING.—Table 2 shows the number of days after clonal division and planting on May 1 until first anthesis in each strain in each series. There was a tendency in any one treatment for southern strains to be delayed in flowering in comparison with northern ones. This tendency was most consistent in series *G*, in which all clones were most vigorous and in which most clones of all strains flowered. In this series strain 1 from Texas came into flower 65 days later than strain 12 from North Dakota.

In five out of the seven strains flowering in both the *N* and the 15-hour series, flowering was earlier on natural daylength. These data suggest a delay in the time of first flowering in these strains on the 15-hour photoperiod. This was brought out more clearly when the comparison of the two series was based on the flowering data of individual plants rather than on the earliest representatives in each strain. There was no consistent tendency for the 14-hour photoperiod either to delay or to accelerate flowering in comparison with the *N* series for the five southern strains flowering on each. In nine strains it required a longer time to first flowering in the *G* series than in the *N* series. In strain 2 the two series flowered at the same time. In strain 12 series *G* flowered first, and there was no flowering in strain 6 in series *N*. It is possible that the delay in series *G* in comparison with *N* may have been related to the average cooler temperature conditions in the garden, which delayed early growth in series *G* and also could have lengthened the time between floral initiation and anthesis.

FLOWERING SEASON.—Extremes in first- and last-flowering dates of each strain and the intervals between are also shown in table 2. One is impressed with the variability among the strains in the length of flowering season in any one treatment. This showed no correlation with latitude. There was also a noticeable tendency for the flowering season of a strain on 14- or 15-hour photoperiod to be longer than in the *N* or *G* series. This was more obvious and significant when the comparison was based on individual plants rather than on those representing the extreme dates in a strain. The data suggest that in some cases the flowering season was lengthened by constant photoperiod or was shortened by the progressively decreasing daylength.

The above suggestion is borne out by the fact that, with the coming of autumn, a state of dormancy was initiated in the *N* and *G* series in which vegetative and reproductive activity ceased in all but the most southern strains, even though the autumn was very mild and frost injury to plants of series *G* was not evident until the middle of November. The plants on 13-, 14-, and 15-hour photoperiods remained green, and those in the 15-hour series continued to produce new elongated tillers. The length of the flowering seasons of some 14- and 15-hour plants was limited by harvesting.

INTERNODAL ELONGATION.—The time required after May 1 for evident elongation of internodes is shown in table 3. Such elongation is sometimes taken as

a criterion of the onset of reproductive activity, but, as shown later, this is not valid in *A. scoparius*. Differences between series in any one strain were probably not significant, except where elongation was completely inhibited on 13- or 14-hour photoperiods. The greatest range between strains was in series *G*, in which strain 8 showed elongation in 68 days; strain 1, in 132 days. In general, it required longer for the southern strains to show elongation than the northern ones.

The length of time between first evident elongation and first flowering (table 3) was more positively correlated with

TABLE 2

FLOWERING SEASON*

Strain no.	No. of days after clonal division on May 1 until first flowering				No. of days between first and last flowering			
	Photoperiod				Photoperiod			
	14	15	*N*	*G*	14	15	*N*	*G*
12	.....	110	124	114	.....	48	1	37
11	.....	121	101	124	.....	16	23	9
10	.....	121	110	119	.....	58	4	18
9	.....	139	121	132	.....	33	30	33
8	.....	105	110	129	.....	60	48	22
7	.....	144	114	124	.....	1	18	21
6	.....	151	.....	144	.....	28	.....	28
5	124	.....	144	148	41	.....	35	24
4	147	151	132	145	32†	28†	19	20
3	132	.....	139	151	40	.....	12	14
2	140	.....	144	144	32	.....	14	28
1	‡	.....	172	179	‡	.....	7	†

Strain no.	First and last dates of flowering							
	Photoperiod							
	14		15		*N*		*G*	
	First	Last	First	Last	First	Last	First	Last
12	.....	.....	8–18	10– 5	9– 1	9– 1	8–22	9–28
11	.....	.....	8–29	9–14	8– 9	9– 1	9– 1	9– 9
10	.....	.....	8–29	10–26	8–18	8–22	8–27	9–14
9	.....	.....	9–16	10–19	8–29	9–28	9– 9	10–12
8	.....	.....	8–13	10–12	8–18	10– 5	9– 6	9–28
7	.....	.....	9–21	9–21	8–22	9– 9	9– 1	9–21
6	.....	.....	9–28	10–26	.....	.....	9–21	10–19
5	9– 1	10–12	.....	.....	9–21	10–26	9–25	10–19
4	9–24	10–26†	9–28	10–26†	9– 9	9–28	9–22	10–12
3	9– 9	10–19	.....	.....	9–16	9–28	9–28	10–12
2	9–17	10–19	.....	.....	9–21	10– 5	9–21	10–19
1	‡	.....	.....	.....	10–19	10–26	10–26	†

* No plants flowered on 13-hour photoperiod.

† Still flowering when harvested.

‡ Floral primordia present on November 1—184 days after clonal division.

strain and treatment. Generally the southern clones required a longer interval than the northern ones in any one series. The number of days between elongation and flowering in general was greater in the 15-hour series than in the *N* series for plants flowering in each. Strain 4 shows the delay on 15 hours quite clearly. In the 14-hour and *N* series it flowered 37 days after initial elongation; in series *G*, in 31 days. On 15-hour photoperiod the interval was 56 days. Such a delay in strain 4 in the 15-hour series and the failure of strains 1, 2, 3, and 5 to flower visibly on this photoperiod suggest that these southern strains have an upper critical limit for flowering at around 15 hours or would be much delayed in flowering on photoperiods of this or greater lengths. It may be recalled that a few culms on some plants of strains 2 and 5 on 15-hour photoperiod showed floral primordia when dissected on November 1. Accordingly, strains 1–5 should probably be classed as intermediate-day plants rather than short-day, since they failed to flower on 13-hour photoperiod, flowered well on 14-hour photoperiod, and failed to flower or were delayed in flowering on 15-hour photoperiod.

In summary, these data on flowering strongly suggest that (*a*) the five southern strains are intermediate-day plants in their photoperiodic responses and that (*b*) the northern strains may be long-day plants, since they are able to initiate flowers on the long natural photoperiods of June[2] and July in Chicago and flowered on 15-hour constant photoperiod but were inhibited from flowering on 13- and 14-hour photoperiods.

TABLE 3

INTERNODAL ELONGATION*

Strain no.	Length of time to first evident elongation after May 1				Length of time between first evidence of elongation and first flowering			
	Photoperiod				Photoperiod			
	14	15	*N*	*G*	14	15	*N*	*G*
12	.....	83	110	99	.....	27	14	15
11	.....	100	87	107	.....	21	14	17
10	.....	111	95	107	.....	10	15	12
9	.....	90	95	99	.....	49	20	33
8	.....	76	83	68	.....	29	27	61
7	.....	114	99	107	.....	30	15	17
6	.....	110	124	114	.....	41	.....	30
5	76	76	76	99	48	.....	68	49
4	110	95	95	114	37	56	37	31
3	100	114	95	114	32	.....	44	37
2	100	124	104	99	40	.....	40	45
1	104	104	104	132	†	.....	68	47

* No evident internodal elongation in 13-hour series.

† Floral primordia were present 80 days after evidence of elongation.

FLOWERING BRANCHES.—A flowering shoot in *A. scoparius* usually includes one or more racemes in a terminal inflorescence and four to six or more axillary flowering branches arising from the upper nodes of the culm. The average num-

[2] As shown by experiments carried on in 1947.

ber of such branches on the central culm at final harvest is shown in table 4. In individual cases they had been produced at nearly all the nodes of the elongated culm. In strain 5 even the axillary buds in the lower leaf sheaths often developed into flowering branches. In the other strains these lower buds usually failed to develop.

The number of axillary buds that developed into flowering branches varied with the geographical strains. In general, the southern and East Coast strains produced a greater number of such branches per culm than the northern and western ones. Strains from the extreme North and the extreme South failed to produce as many as the more centrally located strains.

There was a somewhat direct relationship between the height and number of nodes of a culm and the number of flowering branches. The plants in the garden produced more flowering branches than those on any other treatment. In six of the seven strains which flowered in both the *N* and the 15-hour series, those plants on 15-hour photoperiod produced fewer flowering branches than those in the *N* series.

Vegetative responses

Tiller number and elongation.—The most striking effect of photoperiodic treatment was upon the degree of elongation of leaf blades and sheaths and internodes of stems. There apparently is a distinct critical photoperiod above which plants of a strain showed marked elongation of culms and leaves and below which such elongation was exceedingly limited. None of the plants on 13-hour photoperiod showed such elongation; all of them on 15-hour photoperiod did so (table 5, figs. 2–4). The critical photoperiod for elongation in the species is apparently between 13 and 15 hours. On 14-hour photoperiod, plants of the southern strains (1–5) elongated and produced flowers, while those of northern strains (6–12) showed no elongation. Strain 5 originated at latitude 36° N., strain 6 at 39° N.

The plants on 13-hour photoperiod were much like those on other treatments in growth habits for a few weeks after planting. Their rate of height growth then diminished rapidly, and they showed little increase in height through the remainder of the season. The southern strains grew more rapidly than the northern ones at first and continued to grow for several weeks after the northern ones appeared static. The latter showed little increase in height after the first 2–4 weeks on 13-hour photoperiod, even though new leaves continued to form. However, the blades and sheaths and internodes failed to elongate normally. Large buds formed in the axils of the leaf sheaths. The expansion of these buds forced the leaf sheaths open, spreading the two-ranked leaves apart. Some of the

TABLE 4

AVERAGE NUMBER OF FLOWERING BRANCHES PER CENTRAL CULM*

Strain no.	Photoperiod			
	14	15	*N*	*G*
12	.....	3	4	6
11	.....	5	6	5
10	.....	2	5	5
9	.....	5	4	8
8	.....	7	8	11
7	.....	2	5	4
6	.....	6	.....	9
5	10	.....	12	13
4	5	6	6	6
3	8	.....	8	14
2	7	.....	7	14
1	.....	.....	5	7

* Based on flowering plants only.

fanlike rosettes approached a horizontal plane, but more of them oriented themselves at right angles to the rays of the sun (fig. 6).

Individual plants which failed to show elongation all produced large numbers of tillers. Apparently conditions which hinder elongation or floral initiation may favor an increase in total tiller number. In general, the plants which did not flower produced more tillers than those that did. The 15-hour plants, when taken as a whole, produced the smallest number of tillers of all the treatments.

At final harvest, ten of the strains showed a higher percentage of tillers elongated on 15-hour photoperiod than in the *N* series. The excepted strains are strain 7, in which insect injury to plants on 15-hour photoperiod probably retarded elongation, and strain 11, one clone of which did not flower in this series.

NUMBER OF LEAVES.—At the time of harvest, the central culm was removed from each plant, and the total number of leaves on it was recorded (table 6). In some strains there was no obvious correlation between number and photoperiod or the amount of culm elongation. In part this may have resulted from the difficulties in making accurate counts of the leaves on the unelongated culms in the 13- and 14-hour series. Strains 7, 9, and 10, in which these difficulties were not great, definitely had more leaves per culm under the 13-hour treatment, with no elongation, than in the other series. In contrast, in the southern strains 1, 2, 3, and 4, the numbers were lower in the plants on 13-hour treatment in which no elongation occurred than in the same clones in the other series. These southern strains, however, were much taller in the 13-hour series than were the other strains which produced more leaves per central culm. In the other series, in general, the southern strains produced more leaves per culm than the northern ones. Strain

TABLE 5

TILLER NUMBER

STRAIN NO.	AVERAGE NO. OF TILLERS PER PLANT ON NOVEMBER 1					PERCENTAGE OF TOTAL NO. OF TILLERS THAT ELONGATED*			
	Photoperiod					Photoperiod			
	13	14	15	*N*	*G*	14	15	*N*	*G*
12	24	11	17	9	40	.....	35	26	29
11	11	18	21	12	33	.....	20	50	11
10	7	10	10	8	31	.....	27	16	9
9	9†	9†	9	6	39	.....	59	44	45
8	18	14	11	13	14	.....	39	22	51
7	7†	9†	11	15	38	.....	35	63	17
6	27	25	12	32	93	...	66	8‡	27
5	21	11	7	11	14	26	53	27	46
4	21	17	9	19	38	30	58	24	18
3	20	12	9	16	24	27	40	28	58
2	10	7	5	14	13	43	33	16	62
1	9	7	7	8	27	42	44	26	72

* No elongation on 13-hour photoperiod.

† Large buds not counted as tillers.

‡ Plant did not flower.

5 from North Carolina averaged twenty-one leaves on each central culm in series *N*.

GROWTH IN HEIGHT.—Height measurements taken periodically through the summer of 1946 showed that, on 13-hour photoperiod, strains 1–4 reached their maximum height before July 1. On 14-hour photoperiod, strains 1–2 grew in height until October 15; strains 3–5 continued to increase until about September 15 to October 1, while strains 6–12 showed little change in height after July 1. In the other series in the plants which flowered, growth curves tended to begin to level off just prior to first anthesis, and most of these plants reached their maximum height at about the time of full anthesis.

At harvest time the central culm from each pot was clipped at ground level and was measured from the cut end to the tip of the outstretched leaf or inflorescence. The averaged values are given n table 6. The flowering plants were taller than the nonflowering. The shortest plants were in the northern strains 5–12 on 13-hour photoperiod. Southern strains 1–4 in this series were taller, even though they failed to show marked internodal elongation or to flower. On 14-hour photoperiod, strains 1–5 were very tall, but strains 6–12 were about the same height as on 13-hour photoperiod (figs. 2, 3). In general, all strains were much taller in the 15-hour, *N*, and *G* series than on 13-hour

FIG. 6.—Strain 12 from North Dakota; characteristic rosette condition developed in northern strains grown on 13-hour photoperiod. Leaves continued to form even though blades, sheaths, and culms failed to elongate normally. (Photographed September 29, 1946.)

TABLE 6

CENTRAL CULMS; DATA OF NOVEMBER 1

STRAIN NO.	AVERAGE NO. OF VISIBLE LEAVES					AVERAGE MAXIMUM HEIGHT (CM.)				
	Photoperiod					Photoperiod				
	13	14	15	*N*	*G*	13	14	15	*N*	*G*
12	8.5	11.1	8.5	9.7	8.0	18	15	103	58	78
11	8.8	8.0	10.4	11.2	7.5	21	24	52	109	68
10	11.5	9.3	10.3	7.3	4.9	18	22	47	47	44
9	17.3	12.4	11.6	11.3	11.9	36	49	93	77	95
8	12.0	9.3	11.6	12.0	13.0	23	32	70	77	95
7	18.7	9.0	9.7	10.0	7.3	18	31	90	108	60
6	8.7	7.3	13.2	7.5	11.0	39	30	107	43†	69
5	11.2	13.2	18.3	21.3	17.3	26	107	100	121	98
4	7.3	11.5	13.0	13.1	10.2	49	69	97	112	55
3	8.0	12.8	10.3	15.5	17.0	63	100	53*	108	95
2	8.3	15.4	15.9	13.3	16.4	55	106	86	103	77
1	8.0	15.8	11.8	13.8	14.3	82	119	110	99	89

* Plant weak. † Plants failed to flower.

photoperiod. The various clones of a strain and series in the greenhouse were more variable in height than in the garden.

TOP WEIGHT.—At harvest time the tops were clipped just above soil level, and oven-dry weights were determined (table 7). In all strains except 7 and 11, plants of series *G* yielded more than the comparable plants in any of the other four series. This probably was related in large part to the limiting effect of reduced light intensity in the greenhouse upon the four series grown indoors as well as to the effect of pot culture. The degree of this limitation was most striking in strain 9 from the Indiana sand dunes. However, differences in yield among strains and in relation to photoperiodic treatment were obvious in the greenhouse plants. The southern strains, in general, yielded more than the northern ones in all series. The southern plants are more robust and grow vegetatively for a longer time. In their native habitats they have a longer frost-free period. Strain 9 from Indiana Dunes State Park, however, produced more dry weight than any of the others in series *G* and stood in second or third rank in the 13-, 14-, and 15-hour series. Apparently this strain, native to open sterile dune sand, is able to respond vigorously to improved soil and water relations.

TABLE 7

AVERAGE DRY WEIGHT (GM.) PER PLANT OF CLIPPED TOPS, NOVEMBER 1, 1946

STRAIN NO.	PHOTOPERIOD				
	13	14	15	*N*	*G*
12	24.7	23.5	49.0	18.5*	113.0
11	18.0	20.0	45.9	59.1	52.3
10	12.9	17.1	24.8	20.5	37.0
9	53.7	57.7	81.8	61.0	344.8
8	21.9	26.2	45.0	35.6	92.0
7	5.0	39.7	36.0	96.7	83.0
6	53.0	22.0	117.4	56.2	231.7
5	29.5	57.7	64.6	73.5	123.7
4	43.5	52.1	57.8	81.4	91.2
3	62.2	47.0	29.5*	80.2	180.0
2	52.5	78.9	51.3	67.0	253.4
1	55.6	89.2	79.7	55.3	327.8

* Plants weak.

In many strains the effect of photoperiod upon dry weight was striking. Strains 1 and 2 yielded more in the 14-hour series than in the 13-hour, 15-hour, or *N* series. Many of the northern strains produced only about half as much weight on 13-hour photoperiod as they did on 15 hours. They are usually dormant under natural conditions when daylengths are 13 hours or lower. Some of them also yielded more on the 15-hour treatment than in the corresponding *N* series. This may be correlated with the effect of the former in inducing continued vegetative and reproductive activity, while the plants in the *N* series became relatively dormant after flowering on the decreasing natural daylength.

It should be emphasized that the differences in dry-weight yield among the 13-, 14-, and 15-hour series are an expression of photoperiodic effects and cannot be related to the differences in total irradiation as affecting opportunity for photosynthesis and carbohydrate accumulation. The three series were all exposed equally to 9 hours of natural light per day and differed only in their lengths of exposure (4, 5, or 6 hours) to supplementary illumination of an intensity too low to be very effective in carbohydrate synthesis.

Discussion

The results have shown that *A. scoparius* as a species is strikingly sensitive to photoperiod. It has also demonstrated conclusively that there is considerable differentiation within this widely

ranging species as to the nature of the photoperiodic response. Somewhat similar differentiation in side-oats grama has previously been reported by OLMSTED (19).

The data for 1946 show that culm elongation, dry-weight yield, and floral initiation are sharply controlled by the photoperiod upon which the plants are grown. There is a critical lower photoperiod below which dry-weight yield is low, culms do not elongate, and floral initiation is lacking. For strains of latitude 36° and southward, this critical photoperiod lies between 13 and 14 hours; for strains of latitude 37° and northward, between 14 and 15 hours. Above this critical photoperiod, all strains showed culm elongation and relatively vigorous vegetative growth. Additional experiments in 1947 indicated that in many strains an 18-hour photoperiod tended to induce earlier culm elongation than did a 15-hour one or than did natural daylength at Chicago.

All these data suggest a long-day status of the species. That this is not true of all the strains, however, is shown by the fact that strains 1, 2, 3, and 5 either failed to flower or were much delayed in flowering on 15-hour photoperiod in comparison with a 14-hour one. For floral initiation and vigorous and rapid flowering, they seem to require a narrow range of photoperiods lying between 13 and 15 hours. They would thus be classed as intermediate-day plants. Similar findings were made by ALLARD (1), by ALLARD and EVANS (2), and by ALLARD and GARNER (3) in related species or genera. CORNELIUS (10) found in his collection of strains of *A. scoparius* grown at Manhattan, Kansas, that the southern ones were delayed in flowering until the short days of autumn and were likely to be injured by frost while still vigorously growing. In their native latitudes they experience photoperiods favorable to floral initiation early enough to have flowered and entered dormancy before any danger of frost but in Kansas were not exposed to such photoperiods until August or later.

An intermediate-day status for the northern strains (6–12), based on flowering responses, has not been conclusively demonstrated, and they may probably be classed as long-day plants with a critical photoperiod between 14 and 15 hours. One clone each of strains 6–12 flowered on 18-hour photoperiod in 1947 but were somewhat delayed in doing so in comparison with the same clonal divisions on natural daylength or on 15-hour photoperiod. It is very likely that the northern strains flower normally over a wider range of photoperiods than do the southern ones. The time of onset of flowering in them, in July or August, in their native habitats suggests that these northern strains initiate flowers during the season of long natural photoperiods in June and July. The experimental data indicate in part that possibly the natural decreasing photoperiods are more favorable for the later development of inflorescences than are constant long ones. The experimental results also lead one to the conclusion that the decrease in natural daylength below a certain level may be partially responsible for the cessation of vegetative and reproductive activity and the onset of dormancy, especially in the northern strains.

Since the great majority of species of *Andropogon* are tropical or subtropical in distribution, it is very likely that *A. scoparius* originated in low latitudes in which an intermediate- or short-day response would be typical. It is likely that the northward migration of the species into the higher latitudes of the United

States took place along at least two routes: through the central grassland region and up the Atlantic coastal plain.

On the basis of morphological similarities and dissimilarities, it seemed possible to separate the twelve strains used into three groups: northern (strains 7, 10, 11, 12), central (strains 3, 4, 6, 9), and southern or eastern (strains 1, 2, 5, 8). It will be noted by referring to figure 1 that these morphologically differentiated groups are not correlated solely with latitude but with altitude, physiographic provinces, and vegetational regions also, and, for the central and eastern and southern groups, with the suggested routes of migration. It is possible that the groups are genetically differentiated from one another by reason of isolation of environment. Within each group there may have been a greater chance for genetic interchange than between groups. If this hypothesis is correct, it is interesting that two of the three groups include both intermediate- and long-day strains, suggesting the evolution of the long-day response along both of the suggested routes of migration northward. Since the species is a warm-season grass in accord with its probable origin in low latitudes, a northward migration would have been dependent upon the evolution of the long-day or day-neutral habit in the migrating populations, or at least upon an increase in length of the upper critical photoperiod above that now found in strains 1, 2, 3, and 5. Only in this way could the species have become adjusted to flower during the short growing seasons of the northern latitudes while still retaining the high minimum-temperature requirements for growth still characteristic of it.

Summary

1. Forty plants of *Andropogon scoparius* from twelve points of origin were assembled at the University of Chicago greenhouses in the spring of 1946 and were numbered as shown in figure 1. The twelve geographical strains represented a latitudinal range of approximately 21° from Jackson County, Texas, to Price, North Dakota, and an east-west range from North Haven, Connecticut, to Cheyenne, Wyoming. Their responses to natural daylengths in greenhouse and garden and to constant photoperiods of 13, 14, and 15 hours in the greenhouse were investigated.

2. Growth and development were found to be sharply sensitive to length of photoperiod. None of the twelve strains flowered on 13-hour photoperiod. Plants of only southern strains 2, 3, 4, and 5 produced visible inflorescences on the 14-hour light period. Strain 1 had initiated floral primordia in this series when harvested on November 1. In the 15-hour series, strains 4 and 6–12 produced flowers, but the southern strains 1, 2, 3, and 5 had not visibly flowered at harvest time, even though culms were elongated. Representatives of nearly all the strains produced visible flowers on natural daylength, the southern ones later than the northern. Strains 1–5 should probably be classed as intermediate-day plants rather than short-day, since they failed to flower on 13-hour photoperiod and also failed to flower or were delayed in flowering on 15-hour photoperiod. The northern strains may be long-day plants, since they are able to initiate flowers on long photoperiods but were inhibited from flowering on 13- and 14-hour photoperiods.

3. There apparently is a distinct critical photoperiod above which plants of a strain showed marked elongation of culms and leaves and increase in dry weight and below which such elongation or increase was exceedingly or somewhat limited. None of the plants on 13-hour photoperiod showed elongation; all of

them on 15-hour photoperiod did so. On 14-hour photoperiod, plants of the southern strains (1–5) elongated and produced flowers, while those of northern strains (6–12) showed no elongation. Strain 5 originated at latitude 36° N., strain 6 at 39° N.

4. With the coming of autumn a state of dormancy was initiated in the two series on natural daylength; vegetative and reproductive activity ceased in all but the most southern strains. Plants on 13-, 14-, and 15-hour photoperiods remained green, and those in the 15-hour series continued to produce new elongated tillers. At final harvest, in general, the strains showed a higher percentage of tillers elongated on 15-hour photoperiod than in the indoor series on natural daylength.

The assistance of those who supplied the clonal material and the help of Dr. CHARLES E. OLMSTED in carrying out this experiment are gratefully acknowledged.

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SOME EFFECTS OF 2,4-DICHLOROPHENOXYACETIC ACID ON FRUIT-DROP AND MORPHOLOGY OF ORANGES[1]

W. S. STEWART[2] AND L. J. KLOTZ[3]

Introduction

Recent work has indicated that 2,4-dichlorophenoxyacetic acid (2,4-D) may be superior to other growth-regulating substances for the prevention of preharvest drop of certain varieties of apples (**2**). As with other compounds of this type, its effectiveness in increasing tomato fruit-set has also been demonstrated (**12**). In Florida (**5**) naphthaleneacetic acid and naphthaleneacetamide, when applied in aqueous sprays several months before fruit-drop is expected, have been shown to decrease preharvest drop of pineapple oranges; when applied at the time of fruit-drop, they did not decrease the drop. These sprays have not come into commercial use because growers prefer to risk fruit loss rather than to incur the possibly unnecessary expense of spraying several months in advance of anticipated fruit-drop. Tests with these two chemicals in California were likewise unsuccessful in decreasing preharvest drop of Washington Navel oranges, when applied after the drop had begun. Applications of a wide variety of growth substances also failed to increase fruit-set of Washington Navel oranges or of grapefruit (**9**). For these reasons, problems of fruit abscission in southern California are again being studied to determine the possible use of 2,4-D and related compounds in decreasing fruit-drop of oranges.

PERIODS OF FRUIT-DROP.—Abscission of orange fruit is not restricted to the dropping of matured fruit but may occur at any time during fruit development; in fact, it may be considered a continuous process. There are usually three periods, however, during fruit growth, when abscission may be intense. The first is the period of fruit-set, which usually lasts for a month following full bloom. Generally, sufficient fruit remains on the tree during this period so that prevention of abscission is not an acute problem. There are times, however, when it would be desirable to increase fruit-set.

The second period of intense fruit abscission may occur at the onset of hot summer weather and is referred to as "June drop" (**3**). It is particularly severe with Washington Navel oranges growing in the inland districts of California. At the time of this drop, the young fruit is usually 1.5–2.3 cm. in diameter. Valencia oranges may also lose some of their young fruit during this period, although the drop is less than with Navels.

The third period of intense fruit abscission is called "preharvest drop." This may occur any time after the fruit begins to lose its green color and turn yellow and then orange. With both Valencia and Navel oranges this change in coloring takes place in the late fall, concurrently with the beginning of low night temperatures. Valencia oranges are not picked, however, until the following summer, as

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they do not attain the minimum soluble solids-to-acid ratio of 8 to 1 until that time. It is sometimes even desirable to hold the fruit on the trees for the fall market. Navel oranges, on the other hand, may attain the 8-to-1 ratio by December of the same year in which they flower and are accordingly harvested during the winter. The wet, cloudy weather and severe winds of the winter season hasten the natural fruit-drop of both varieties. Hot summer weather, which increases the June drop of immature fruits, also seems to increase the loss of mature Valencia oranges.

FIG. 1.—Curling of young, expanding Valencia orange leaves in response to aqueous sprays (*left to right*) of 0, 15, and 30 p.p.m. of 2,4-D. (Photographed 10 days after spraying.)

The present paper reports some of the effects of 2,4-D on fruit abscission during the three periods described above.

Experimentation

All the 2,4-D applications referred to in this paper were made as water sprays, using diethanolammonium 2,4-dichlorophenoxyacetate, which was obtained as a commercial liquid preparation containing the equivalent of 40% free 2,4-D. No supplementary spreading agent was used. The spray equipment varied from 3-gallon, continuous-pressure, Hudson-type garden sprayers to 600-gallon, high-pressure spray rigs. Concentrations of 2,4-D were varied from 1 to 240 p.p.m. The higher concentrations used in these tests with 2,4-D (60–240 p.p.m.) were much higher than those employed in previous tests with naphthaleneacetic acid and naphthaleneacetamide in Florida (**5**), because even the highest concentrations of those two chemicals, in previous tests in California, had failed to decrease fruit abscission. Since the tolerance of orange trees to 2,4-D was not known at the beginning of the present studies, usually only one-quarter or one-half of a tree was sprayed.

FRUIT-SET.—To determine whether 2,4-D would increase fruit-set, two field plots of Valencia orange trees were established—one plot at East Covina and the other at Glendora, California.

At the East Covina plot, concentrations of 5, 15, 30, and 60 p.p.m. of 2,4-D were tested. At each concentration the spray was applied to the east half of one tree and to the west half of another. Applications were made on April 19, 1946, during the period of full bloom. Mature leaves showed no apparent response, but within 24 hours all treatments had induced a downward and inward rolling of the margins of the young, expanding leaves (fig. 1). The response of the young

leaves was most pronounced at the highest concentrations of 2,4-D and decreased with decreasing concentrations so that it was barely perceptible with the 5-p.p.m. spray. Two to 3 weeks after spraying it became apparent that the treatment had increased the number of twigs from sprayed trees, the ovary, stamens, and corolla in nearly every flower remained securely attached to the receptacle, whereas on the unsprayed twigs a large percentage of the flowers dropped, and of other flowers only the ovary remained.

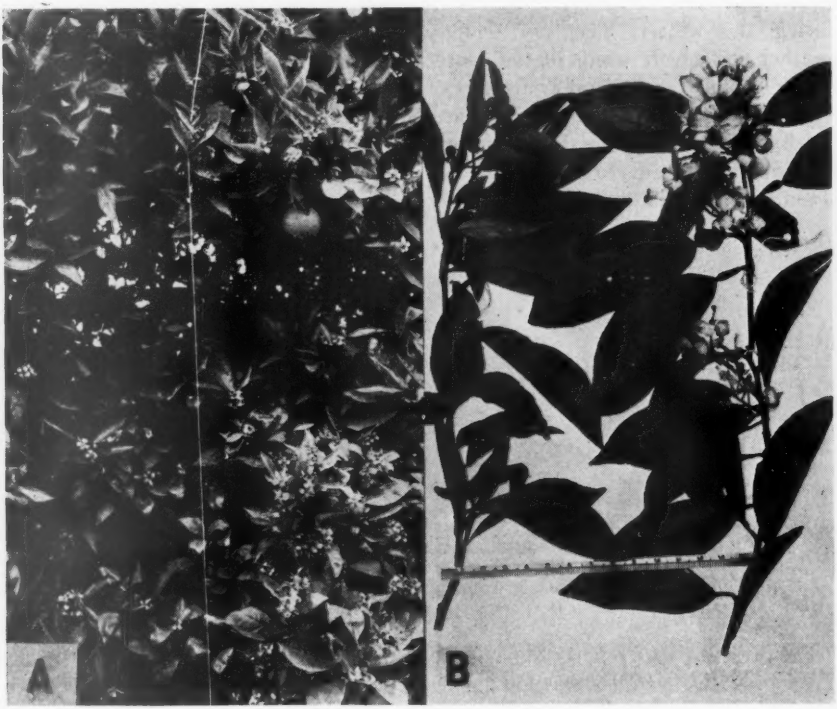

FIG. 2.—Effect of aqueous spray of 60 p.p.m. of 2,4-D applied to Valencia orange tree at full bloom, April 19, 1946. *A*, *left*, not sprayed; *right*, sprayed. *B*, representative twigs from unsprayed (*left*) and sprayed (*right*) portions of same tree. (Photographed May, 1946.)

flowers retained on the trees (fig. 2, *A*, *B*).

As is well known, ethylene vapors greatly accelerate the rate of abscission of leaves, flowers, and fruits. When twigs from the sprayed and unsprayed trees were exposed to ethylene in the laboratory, they showed that the 2,4-D had within 1 week effectively decreased flower and leaf abscission (fig. 3). On the

In the field the complete flowers were still retained on the sprayed trees 8 weeks after spraying. It became evident at this time, however, that, although the flowers had not dropped, the ovaries were pale yellow in color instead of the usual deep green, and relatively few were developing into fruit. Furthermore, the ovaries failed to enlarge, whereas those on the unsprayed trees were two to three

times as large as at the beginning of the experiment. In late May, with the coming of summer weather, nearly all the flowers that had been retained on the sprayed portion of the trees dropped, and, as a result, with 2,4-D sprays of 30 p.p.m. or more, there appeared to be even fewer young fruits remaining on the sprayed portion of a tree than on the unsprayed. It was also evident from these trials that the blossom-retaining effect of 2,4-D was not transmitted from the sprayed portion of the tree to the unsprayed area.

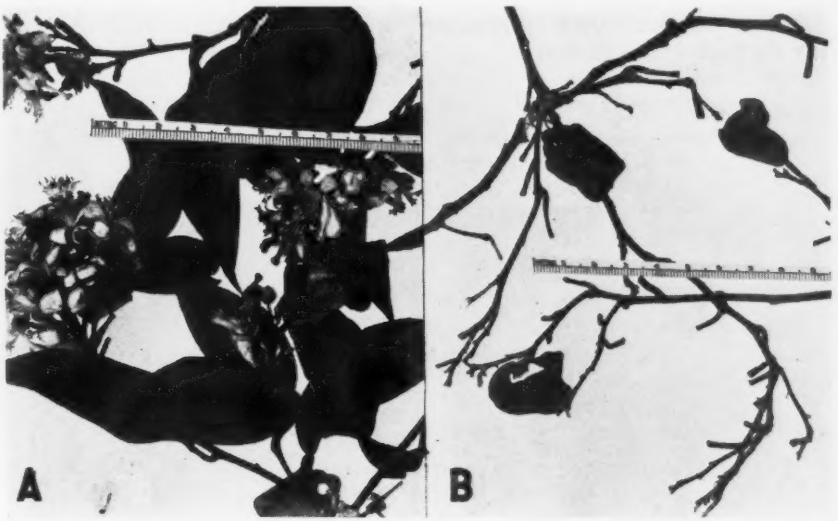

FIG. 3.—Twigs of Valencia orange after 58 hours in ethylene chamber at high humidity: *A*, from portion of tree sprayed 10 days earlier with 60 p.p.m. of 2,4-D; *B*, from unsprayed portion of same tree.

These results were duplicated at the Glendora plot, where concentrations of 15, 30, 60, 120, 240, and 480 p.p.m. of 2,4-D were tested. For each concentration alternate quadrants were sprayed on the north halves of two trees.

It was found that at all concentrations the 2,4-D sprays effectively decreased the usual abscission of ovaries and flower parts for 8–12 weeks, or longer, after application. It seems unlikely that a second spraying during that time would have been any more effective in inducing fruit development than was the first. Although 2,4-D can apparently delay the formation of abscission layers in the flower parts, the effect, in these trials at least, was not such as to stimulate enlargement of the ovary. A different or additional growth factor is probably necessary for the enlargement of the ovary. This might be found in pollen extracts or in the stilbene compound recently reported as being very effective in inducing fruit growth of tomato (11).

JUNE DROP.—Studies of the effectiveness of 2,4-D sprays in decreasing June drop of young orange fruits were begun on May 31, 1946. Aqueous sprays containing 25, 75, and 225 p.p.m. of 2,4-D were applied on trees of both Washington Navel and Valencia oranges at Riverside. The Navel plot consisted of six randomized blocks in which east or west halves

of the trees were sprayed, the sides alternating from block to block. The Valencia plot consisted of a Latin-square arrangement of trees, which were sprayed on alternate east or west halves, according to the row. Immediately after spraying, all the leaves and young fruit were removed from underneath the trees, and a heavy circular wire hoop inclosing an area of 10 square feet was placed under the sprayed half of each tree. Subsequent fruit-drop was determined by counting only the fruit within the hoop.

readily with transmitted light. This response was not observed in the leaves of the Navel orange but was noted in leaves on grapefruit trees sprayed with this concentration of 2,4-D (**10**).

On both Valencia and Navel orange trees, the 75- and 225-p.p.m. sprays decreased the amount of maturing fruit and induced morphological changes in the fruit still remaining on the trees. The 225-p.p.m. treatment on the Valencia trees caused a curious pebbling of the peel of the green fruit (fig. 4). Prelimi-

TABLE 1

EFFECT OF AQUEOUS SPRAYS OF 2,4-D ON JUNE DROP OF YOUNG WASHINGTON NAVEL AND VALENCIA ORANGES. SPRAY APPLIED ON MAY 31, 1946 FIRST FRUIT COUNT, JUNE 13; SECOND COUNT, JULY 12, 1946*

CONCENTRATION OF 2,4-D (P.P.M.)	AVERAGE NO. OF FRUITS DROPPED PER TREE					
	Navels			Valencias		
	First count	Second count	Total	First count	Second count	Total
0 (unsprayed control)	52	83	135	134	105	239
25	61	106	167	68	177	245
75	33	149	182	93	149	242
225	19	124	143	49	158	207

* In area of 10 square feet under each tree.

In both plots the 2,4-D delayed abscission of the young fruit for 6–8 weeks, but after that time the fruit dropped to the same extent as though the trees had not been sprayed (table 1). In addition, there was severe curling of all foliage expanding at the time of the spray application. Actually, however, as the treatment was made between flushes of leaf growth, little response of this type was observed. The mature leaves of the Valencia orange trees sprayed with 225 p.p.m. of 2,4-D developed chlorotic areas. These were usually found in the tissue between the lateral veins and could be seen most

nary microscopic observations of free-hand sections indicate that this is the result of both outward and inward elongation of the oil glands of the peel. These glands in normal fruits are usually ellipsoidal and imbedded in the peel and do not protrude above the surface of the rind as in the sprayed fruits (**6**). The rind was also several times thicker on the sprayed fruits than on the unsprayed.

On the same Valencia trees, some fruits became cylindrical in shape and developed a small navel complete with juice vesicles (fig. 4). The spherically shaped fruits on either treated or un-

treated trees showed no comparable navel structure but only the presence of undeveloped vascular strands in the stylar portion. Navel development, cylindrically shaped fruit, and pebbled and thickened peel were likewise found in grapefruit in response to an aqueous spray containing 225 p.p.m. of 2,4-D, applied on June 3, 1946, to trees bearing young fruit (**10**).

Young Navel fruits sprayed with 225 p.p.m. of 2,4-D showed somewhat less tendency toward pebbled peel than the Valencia fruits but developed a thicker rind than did unsprayed fruit. The sprayed fruit also either developed excessively large, protruding navels or grew into cylindrically rather than spherically shaped fruit (fig. 5), suggestive of that produced on trees affected with "stubborn" or "acorn" virus disease (**3, 5**). A few of the unsprayed fruits developed protruding navels, but nearly all the sprayed ones had this characteristic. In addition, all the sprayed fruits examined had seeds or seedlike structures (fig. 6), whereas the unsprayed fruits were seedless, as is usual with this variety of orange. The seeds varied in length from less than a millimeter to over a centimeter, the largest being in fruits on trees sprayed with 75 or 225 p.p.m. of 2,4-D.

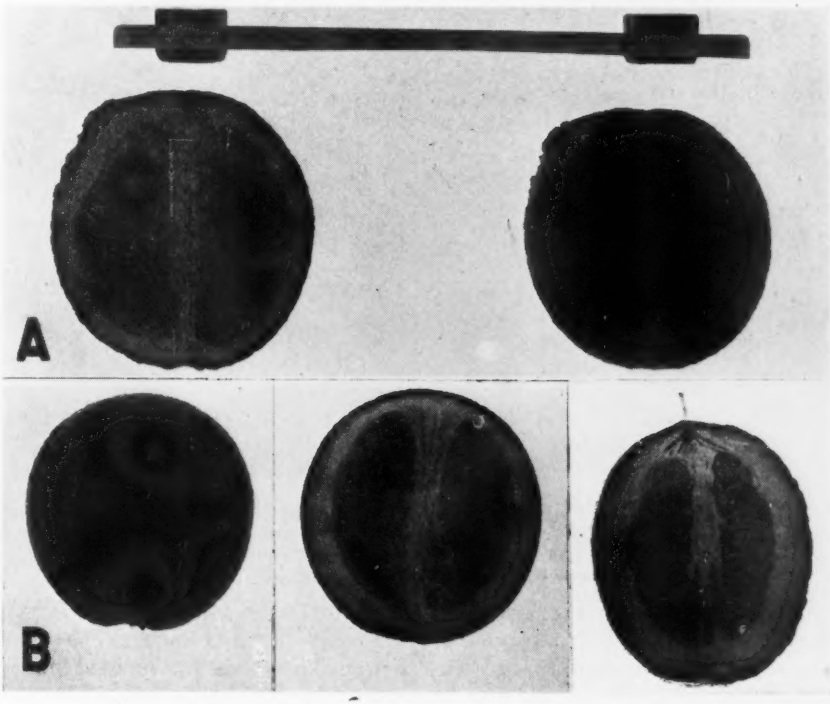

FIG. 4.—*A*, pebbled appearance of rind of fruit of Valencia orange tree sprayed with 75 p.p.m. of 2,4-D on May 31, 1946. (Photographed October 14, 1946.) Note navel structure and rind proliferation where fruit touched ground. *B* (*left*) spherical (normal) shape of fruit from unsprayed control tree; (*right*) cylindrical shape of fruit from tree sprayed with 225 p p.m. of 2,4-D. (Photographed November 1, 1946.) Note persistent style and navel structure.

Dissection of a hundred of the largest seeds showed that most of them had embryos, of which only about 15% were over 3 mm. in length. Cultures are being attempted with the largest embryos. Seed counts of seventeen fruits from the trees sprayed with 225 p.p.m. of 2,4-D indicated an average of 53.9 seeds per fruit, with a standard error of ±7.1. The different responses of the young Navel fruits to high-concentration sprays were probably the result of spraying fruit in slightly different growth stages. As none of these changes in fruit morphology was anticipated, individual fruits were not tagged at the time of spraying.

On January 9, 1947, the fruits on the sprayed and unsprayed Navel trees were harvested and counted. Fruit size was determined by counting the fruit in each full field box. It was found that the 25-p.p.m. spray had increased fruit size, compared with that of unsprayed fruit and that still greater size increases had been obtained in fruit sprayed with 75 or 225 p.p.m. of 2,4-D (table 2). These last two concentrations also resulted in a decreased fruit yield. It is possible, however, that the increases in fruit size were a direct response to the 2,4-D itself and not an indirect response to the fruit-thinning effect of reduced yield, since

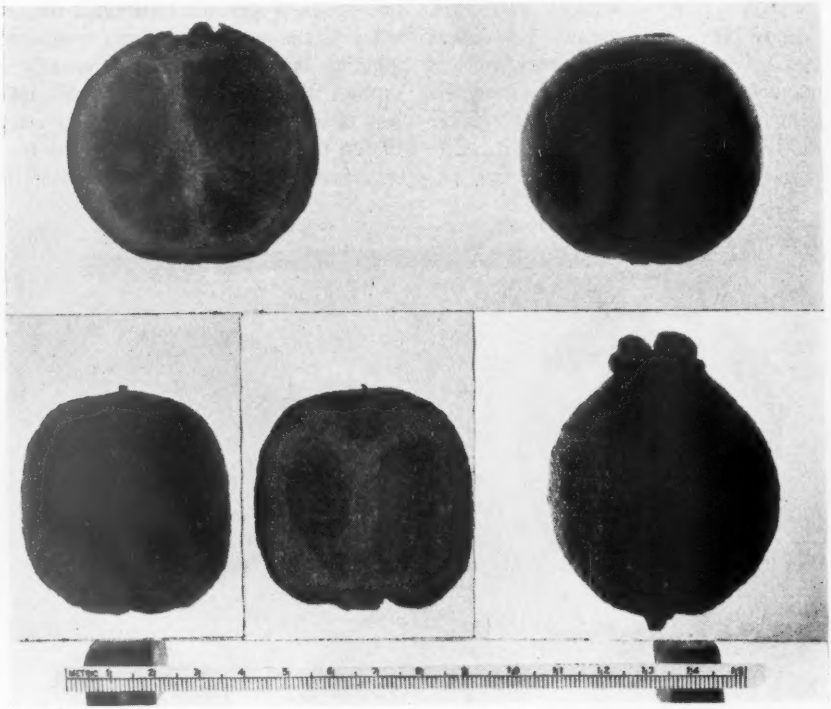

FIG. 5.—Washington Navel oranges. *Above*, normal fruit from unsprayed portion of tree. *Below* (*left*), cylindrical shape of some fruit and (*right*) excessive navel growth of other fruit from portion of same tree, to which aqueous spray of 225 p.p.m. of 2,4-D had been applied.

hand thinning of Navel oranges results in diameter increases of not more than 8% (**8**), compared with increases of as much as 25% obtained with the sprays. These data suggest a possible use of 2,4-D sprays for increasing fruit size.

At the time of obtaining the above yield records, composite samples of a hundred and twenty fruits (twenty from the treated half of each tree) were taken for quality studies. The fruit samples were selected to be representative of the treatments; they were not random samples. Physical measurements confirmed the results of field-box counts and indicated that the treated halves of the trees produced larger fruits than the untreated. They also showed that changes in fruit structure accompanied size increases. On the basis of fruit weight, there was an increase in percentage of rind and rag, and a decrease in juice, with increasing concentrations of 2,4-D (table 3). Chemical analyses of the juice indicated a decrease in total acids as a result of the sprays (table 4). Since large fruit has been found to contain a lower concentration of soluble solids than small fruit (**1**), the slight decreases in soluble solids observed in the large fruit from the treated halves of the trees are not considered significant. The very

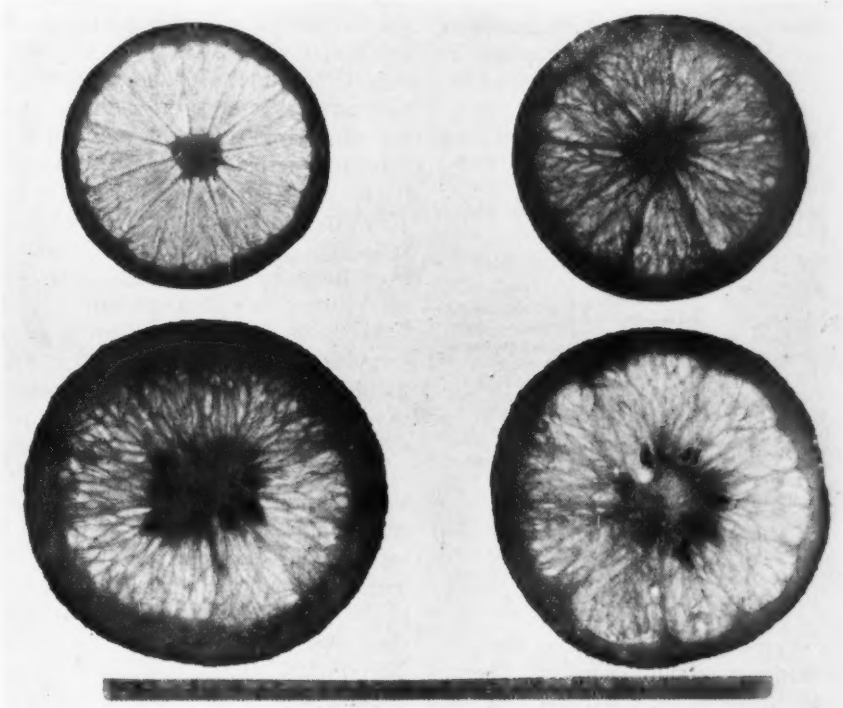

FIG. 6.—Washington Navel oranges showing seedlike structures in fruits from trees sprayed on May 31, 1946, with the following amounts of 2,4-D in water: (*upper right*) 25 p.p.m., (*lower left*) 75 p.p.m., (*lower right*) 225 p.p.m. Fruit at upper left is an unsprayed control. (Photographed November 1, 1946.)

slight decreases in ascorbic acid are likewise not considered significant.

The effectiveness of 2,4-D sprays in decreasing June drop of oranges appears to be in essentially the same category as its effectiveness in increasing fruit-set. In both cases the drop was delayed, only to be followed by the same amount of drop as though the spray had not been applied. In fact, at concentrations of 2,4-D above 25 p.p.m., the sprays induced greater delayed drop, so that production was actually decreased. The increase in size of the remaining fruit suggests possible use of these sprays to increase fruit size; however, at the expense of commercial quality. Although satisfactory results for the control of June drop were not obtained, there are indications from the data that the problem may be solved by further studies in this direction.

TABLE 2

EFFECT OF AQUEOUS SPRAYS OF 2,4-D ON YIELD AND SIZE OF WASHINGTON NAVEL ORANGE FRUITS. SPRAYS APPLIED TO ONE-HALF OF EACH TREE ON MAY 31, 1946; FRUIT HARVESTED ON JANUARY 9, 1947

Concentration of 2,4-D (p.p.m.)	Yield (average no. of fruits from halves of six trees)		Size (average no. of fruits per field box, from halves of six trees)	
	Sprayed	Unsprayed	Sprayed	Unsprayed
0 (unsprayed control)	.....	300	.....	163
25	327	256	147	182
75	186	272	111	164
225	73	289	100	137

PREHARVEST DROP.—The effect of 2,4-D on preharvest drop of Valencia oranges was studied in two field plots at Rivera, Los Angeles County, California, and in one plot at Camarillo, Ventura County.

Severe preharvest drop is usually an annual occurrence in the Rivera district, apparently as a result of environmental conditions there. The first plot established in this area consisted of five treatments in six replicated, randomized blocks, one tree in each block being used per treatment. In three of the blocks the east half of the tree was sprayed; in the other blocks the west half was treated. Concentrations of 2,4-D used were: 0, 5, 10, 20, and 40 p.p.m. The second plot consisted of treatments of 0, 5, and 20 p.p.m. of 2,4-D. In this plot, as in the June-drop studies, east or west halves of trees were sprayed, the sides alternating from block to block. There were four randomized trees per treatment.

In both plots, immediately after spraying, all fruit on the ground was removed, and subsequent periodic counts were made of the fruit dropped. In addition, in the first plot, fruit remaining on the tree at the end of the experiment was determined for both treated and untreated halves, so that fruit-drop here is expressed as a percentage of the total number of fruits on the halves of the tree at the beginning of the experiment. This eliminates the effect of tree size on the drop counts.

There was a significant reduction in fruit-drop in both Rivera plots as a result of the 2,4-D sprays. In the first plot this varied from 39.6 to 55.7% for the 5-p.p.m. and 40-p.p.m sprays, respectively (table 5). The decreases in fruit-drop with sprays of 5–20 p.p.m. of 2,4-D were significant at 5%; the decrease with the 40-p.p.m. spray was significant at 1%. In the second plot, where effects of tree size could not be eliminated, the 5-p.p.m. spray failed to decrease fruit-drop significantly; the 20-p.p.m. spray, however,

effected a decrease of 49.3%, which was significant at the 5% level (table 5).

The Camarillo plot was established on September 5, 1946, late in the harvest season for Valencia oranges. There had been a heavy fruit-drop during the preceding 2 weeks, and an estimated two *hundred to three hundred fruits per tree* had already dropped before the treatment was applied. This plot consisted of forty trees, twenty trees in each of two adjacent rows. Ten of the trees in each *row were sprayed with 25 p.p.m. of* 2,4-D, and the others were left unsprayed for comparison. The entire tree was treated. In each row the sprayed and *unsprayed trees alternated, so that, in* comparing the trees in the two adjacent rows, one always found a treated tree opposite an untreated one. Immediately after spraying, all fruits were removed *from the ground under the trees.*

On October 9 the fruit on the ground was counted (fig. 7) and that remaining on the trees was harvested and counted. On the basis of the total amount of fruit *on the trees at the beginning of the ex*periment, there was an average drop of

TABLE 3

EFFECT OF AQUEOUS SPRAYS OF 2,4-D ON PHYSICAL-QUALITY FACTORS OF WASHINGTON NAVEL ORANGE FRUITS FROM HALVES OF TREES SPRAYED ON MAY 31, 1946; FRUITS HARVESTED ON JANUARY 9, 1947*

Concentration of 2,4-D (p.p.m.)	Whole fruit			Rind† (weight)		Rag‡ (weight)		Juice (weight)	
	Volume (ml.)	Diameter (cm.)	Weight (gm.)	Gm.	% of whole fruit	Gm.	% of whole fruit	Gm.	% of whole fruit
0 (unsprayed control)	143.5	6.45	130.7	61.8	47.3	1.39	1.1	67.5	51.6
25	210.6	7.49	178.0	89.9	50.5	5.54	3.1	82.6	46.4
75	261.2	7.63	227.4	122.5	53.9	8.40	3.7	96.5	42.4
225	293.9	8.05	256.9	137.5	53.6	13.80	5.4	105.6	41.0

* Composite samples of 120 fruits each.

† Flavedo, albedo, and any additional attached tissues remaining after reaming fruit on electric juice extractor.

‡ Tissues not passing through vibrating sieve on electric juice extractor.

TABLE 4

EFFECT OF AQUEOUS SPRAYS OF 2,4-D ON CHEMICAL-QUALITY FACTORS OF WASHINGTON NAVEL ORANGE FRUITS FROM HALVES OF TREES SPRAYED ON MAY 31, 1946; FRUIT HARVESTED ON JANUARY 9, 1947*

Concentration of 2,4-D	pH	Total acid, as citric (%)	Soluble solids (%)	Ratio of soluble solids to total acid	Ascorbic acid (mg./100 ml. juice)
0 (unsprayed control)	3.29	1.37	12.72	9.28:1	61.53
25	3.29	1.21	12.58	10.40:1	60.06
75	3.39	1.15	12.51	10.53:1	57.13
225	3.43	1.14	12.58	11.03:1	58.60

* Composite samples of 120 fruits each.

TABLE 5

PREHARVEST FRUIT-DROP IN TWO PLOTS OF VALENCIA ORANGE TREES AT RIVERA, CALIFORNIA ALTERNATE HALVES OF TREES SPRAYED WITH AQUEOUS SOLUTIONS OF 2,4-D ON MAY 17, 1946

Concentration of 2,4-D (p.p.m.)	Plot 1						Plot 2	
	Percentage of fruits dropped May 17 to August 10, 1946 (average from halves of six trees)*						No. of fruits dropped, May 17 to July 8, 1946 (average from halves of four trees)	
	Computed from trees in randomized blocks			Computed from paired comparison of sprayed and unsprayed halves of same tree				
	Unsprayed	Sprayed	Decrease due to spraying	Unsprayed	Sprayed	Decrease due to spraying	Unsprayed	Sprayed
0 (unsprayed control)	10.6†						24.5	
5		6.4	39.6	9.27	6.37	31.3‡	29.5	28.5
10		7.5	29.3	10.79	7.53	30.2‡		
20		6.0	43.4	10.40	5.95	42.8‡	22.3	11.3§
40		4.7	55.7	12.27	7.61	38.0‖		

* Percentage of total fruit on trees at time of spraying.

† Least significant difference at 5% level, 2.49; at 1% level, 3.36.

‡ Significant at 5% level.

§ Significant at 5% level by *t*-test, in comparison with average fruit-drop (25.6) based on the sixteen unsprayed tree halves.

‖ Significant at 1% level.

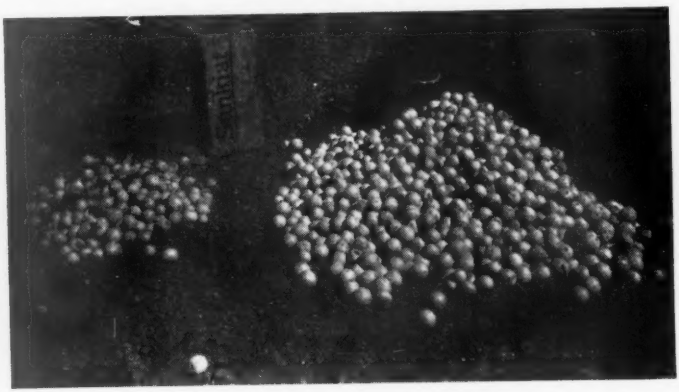

FIG. 7.—Valencia oranges dropped between September 5 and October 9 at Camarillo plot: (*left*) from twenty trees sprayed with 25 p.p.m. of 2,4-D and (*right*) from twenty unsprayed trees.

11.05% from the unsprayed trees, compared with 2.47% from the sprayed ones. This difference of 78% is significant at the 1% level. The number of fruits dropped from the twenty unsprayed trees was 2,861, compared with 599 from the sprayed trees. In addition, most of the fruit dropped from the unsprayed trees was firm and of a better quality than that dropped from the sprayed ones. The spray treatment thus not only decreased drop but also resulted in a drop of only the poor-quality fruit.

Fruit-quality analyses of representative composite samples of one hundred fruits each from twenty unsprayed and from twenty sprayed trees indicated no significant differences in soluble solids, total acid, pH, or ascorbic acid.

With the preharvest sprays, as with the fruit-set and June-drop sprays, it was observed that a curling of the expanding young leaves occurred at concentrations of 2,4-D above 5 p.p.m. At 5 p.p.m. leaf curling was barely perceptible. When the sprays were applied between leaf-growth flushes, little or no curling of leaves was apparent.

In the plots at both Rivera and Camarillo the treatments were applied after fruit-drop had started. GARDNER (**5**), in Florida, reported that, in the use of sprays of naphthaleneacetic acid and naphthaleneacetamide, the more in advance of the drop the application was made, the less was the drop. When applied 3–4 months before the drop, they induced the greatest decrease; their application when fruit was dropping caused little or no decrease in fruit-drop. In contrast, the 2,4-D sprays decreased fruit-drop as much as 78% when applied even after the drop had been occurring for 2 weeks. At present, the main difficulty in the use of 2,4-D sprays is their curling effect on new foliage. It is not known to what extent, if any, curling may impair the efficiency of the leaves or be a serious objection. The spray would be applied only once a year for preharvest fruit-drop, whereas there are at least three leaf-growth flushes a year. For this reason, only one set of new leaves would be affected, and if the spray were applied between growth flushes even this amount of leaf injury might be avoided.

Summary

1. Some effects of aqueous sprays of 2,4-dichlorophenoxyacetic acid (2,4-D) on fruit-drop and morphology of oranges are reported.

2. When applied to Valencia orange trees at full bloom, these sprays delayed blossom-drop 8–10 weeks, or more, but did not increase fruit-set. Applications in May likewise delayed the June drop of immature Washington Navel orange fruits 6–8 weeks but did not increase yield.

3. Young Navel fruits sprayed at this time with concentrations of 25–225 p.p.m. of 2,4-D developed seeds, or seed-like structures, in contrast to unsprayed fruits, which were seedless. Some fruit on the trees sprayed with concentrations of 225 p.p.m. of 2,4-D developed a thick rind and grew excessively large, protruding navels; other fruits became cylindrical in shape.

4. Fruit on Valencia orange trees treated in May with a concentration of 225 p.p.m. of 2,4-D developed a thick, pebbly rind. Other fruit on these trees became cylindrical in shape and developed small navels.

5. Applied to Valencia orange trees bearing mature fruit, the sprays decreased preharvest fruit-drop as much as 78%, even when applied 2 weeks after severe drop had begun.

6. Until additional data are available

from studies now in progress, these sprays are not recommended for commercial use.

The authors appreciate the generous and willing co-operation of the owners of the groves used in this work. They thank the Thompson Horticultural Chemicals Corporation of Los Angeles for furnishing the 2,4-dichlorophenoxyacetic acid. The assistance of Mr. LOUIS BRANNAMAN and Mr. HOWARD EVANS greatly facilitated these studies.

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EFFECTS OF 2,4-DICHLOROPHENOXYACETIC ACID ON GAS EXCHANGE OF WHEAT AND MUSTARD SEEDLINGS

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY **592**

D. L. TAYLOR[1]

Introduction

Information has been contributed by many investigators concerning the nature of responses of various tissues and plants to variations of treatment with the substituted phenoxyacetic acids, especially 2,4-dichlorophenoxyacetic acid (2,4-D). The present wide use and indicated possibilities for even broader uses of these chemicals make it most desirable to obtain more information as to the way, or ways, in which they influence metabolism. Several investigators have reported on the hydrolytic and mobilization responses involving reserves in a number of dicotyledonous species after treatment with chemical growth-regulators (**14**, **15**, **21**, **22**, **26**). Often the amount of mobilized carbohydrate has been observed to decrease before the end

[1] Deceased December 6, 1947.

of the tests or prior to the death of treated plants.

BROWN (**7**), who investigated water relations and the distribution of solid matter in kidney beans, used a volumetric method with an 18-hour test period and found distinctly increased carbon dioxide (CO_2) evolution by beans 5–7 inches tall and by mature morning-glory plants sprayed with 1000 p.p.m. of 2,4-D. By means of manometric technique SMITH *et al.* (**21**) recently observed increased rates of oxygen (O_2) uptake by segments of underground stems of bindweed field-sprayed with 2,4-D. It has been suggested that starvation may have developed as a part of the complex of responses, resulting in the eventual death of treated plants. An interpretation has been made that increased utilization of reserves resulting from stimulated respiratory activity may have contributed toward development of starvation.

Since November, 1946, many tests have been conducted on mustard, radish, wheat, barley, and rice seedlings in a study of effects of 2,4-D on their metabolism of energy release. This paper presents results on the intensity and duration of treatment with 2,4-D as correlated with the alteration of gas-exchange rates of mustard and wheat seedlings at different stages of seedling development.

Material and methods

Certified seed of Henry variety of spring wheat (*Triticum vulgare*) and white mustard (*Brassica alba*) were used. The 2,4-D, recrystallized twice from material of a technical grade, was a small crystalline, white powder, m.-p. 134°–136° C. The rates of O_2 uptake and CO_2 evolution of samples of seedlings which had been variously treated with 2,4-D were measured with standard Warburg manometric equipment, using details of the technique for the direct (two-vessel) method (**27**).

In preparation for a test, sound seeds were stirred 4 minutes in a saturated, filtered solution of calcium hypochlorite. After subsequent thorough stirring in six changes of water, the seeds were spread on two layers of filter paper in moist-chambers 8 inches in diameter which contained 10–20 ml. of free water. Germination progressed in the dark in an incubator at 28° C. for wheat and at 25° C. for mustard until the seedlings were of an age desired for treatment, after which large numbers of uniform seedlings were selected, divided into lots, and each lot placed in the desired treatment solution. The period of germination was always considered to begin at the time of treatment to disinfect seeds.

All manometric measurements were made in the dark at 28±0.1° C. in solutions buffered by *M*/100 monopotassium acid phosphate. In all experiments the manometers were moved 110–120 strokes per minute. It was established that the activity of standard seedling samples in air and in nitrogen did not vary when manometers were moved at more than 90 strokes per minute and that the activity of untreated seedlings increased uniformly in air during test periods lasting 3 hours. The O_2 content of the air in the vessels or the diffusion of gas in the substrate solution (2.5 ml.) apparently did not limit aerobic activity under these conditions.

Manometers were read at intervals of 10–15 minutes during test periods of 45–100 minutes, depending upon the intensity of activity and the size of sample tested. The rates of O_2 uptake and CO_2 evolution and the size of seedlings varied for different kinds and ages. These qualities were factors considered in determin-

ing the numbers of seedlings of different kinds or ages used in manometric tests.

Eight to twenty-five selected seedlings were used in each replicate. The number used per replicate was smaller as the seedlings became older and was larger for mustard than for wheat seedlings of the same age. The same numbers of seedlings of a species were used in the replicates of all treatments in different tests of seedlings of any specific age.

Manometric data were converted to volume values, and the rates of O_2 uptake and CO_2 evolution were calculated (**27**) on a seedling basis. In previous studies on wheat (**19, 24**) and on other cereals (**4, 12, 24**), embryonic activity has been found to account for 80–90%, or more, of the total seedling utilization of O_2 or of CO_2 evolved. Therefore, embryos (minus scutella) were removed from the wheat seedlings tested, dried to constant weight, and the activities calculated on the basis of 1 mg. dry weight of such embryonic tissue.

Mustard seedlings proved to be much less satisfactory for study than wheat. Rates of O_2 uptake and CO_2 evolution were relatively low, and, though larger samples—20 ± 5 seedlings—were tested, the variations in results (up to 10%) were often greater than those for wheat (8% or less). Subdivision of mustard seedlings into relatively active and inactive parts was not possible as it was for wheat. Values for untreated mustard were obtained which were relatively very low when data for some of the initial tests were calculated on the basis of unit of dry weight of seedling tissue (see data for seedlings 70 hours old, table 3). Since no consistent advantage resulted from such calculations, weight data were not obtained in several of the tests, and the results are presented on the basis of seedling samples only.

Several advantages are gained from manometric techniques, even though the number and the size of seedlings (the amount of tissue) and the length of a test period without resetting of the apparatus tend to be somewhat limited. Accurate data could be obtained from numerous, comparatively brief tests (**11**) in which relatively small samples of intact seedlings, or seedling tissues, could be subjected to a variety of conditions of environment and treatment with 2,4-D according to techniques which have been used, fully described, evaluated, and accepted by numerous individuals.

Some advantages in using seedlings, or tissues thereof, were the ease of obtaining ample, uniform test material in minimum time; the normal occurrence of very high activity in such material; and the ease of accomplishing significant variations of treatment. Greater reproducibility of samples of tissue used in a test and between results of different tests and replicates of a treatment was found to be possible if the seedlings were selected for treatment when their early development could be determined to be normal. It should be kept in mind that previous studies have shown that a succession of rather marked qualitative and quantitative changes in activity normally occurs during the development of many seedlings or their parts (**1, 6, 10, 12**).

Experimentation

In a number of initial tests information was obtained concerning the degree of apparent response of the metabolism of energy release in relation to duration of treatment. Some details of technique in later tests were determined in part according to results of these tests. The rate of O_2 uptake was measured for representative, fifteen-seedling samples of wheat

and mustard during the twenty-fourth hour after seeds were disinfected. When seedlings were 24 hours old, buffered 2,4-D solution was tipped from a side-arm into the $M/100$ phosphate buffer in the seedling chamber of the vessel and thoroughly mixed so that the 2,4-D concentrations which resulted were 0, 1, 2.5, or 5 p.p.m. During the first and second hours after addition of 2,4-D the O_2 uptake by the samples was measured (table 1).

Earlier studies (**10, 12**) have shown that marked changes in the rate of respiration parallel to rapid growth occurred in seedlings during a similar period. It would seem possible that the rate of activity of the samples might reflect some such change even during relatively short test periods. The progressive development of the effect of the treatment with 2,4-D on growth (size of sample) might be a factor to be considered in evaluation of the metabolic responses of variously treated samples of seedlings. Treatment of data for gas exchange on the basis of weight of embryonic tissue might facilitate evaluation or elimination of the effect of the acid on growth of the sample. Adjustment for unavoidable

TABLE 1

PROGRESSIVE DEVELOPMENT OF EFFECT OF 2,4-D ON RATE OF O_2 UPTAKE BY WHEAT OR MUSTARD SEEDLINGS 25 HOURS OLD. ACTIVITIES SHOWN AS CU. MM./HR./10 SEEDLINGS

Time of measurement	Treatment (p.p.m.)							
	Wheat				Mustard			
	0	1	2.5	5	0	1	2.5	5
	Measured rate*							
$T-1$	183	174	174	178	35.0	36.0	42.0	53.0
$T+1$	192	168	180	172	41.0	30.0	34.0	59.0
$T+2$	196	162	174	174	37.5	24.0	28.0	50.0
	Change in rate measured†							
$T+1$	+5	−3	+3	−3	+17	−17	−19	+11
$T+2$	+7	−5	0	−2	+ 7	−33	−33	− 6
	Difference between measured and expected rates‡							
$T+1$	.....	− 8	−2	−8	.....	−19	−31	− 5
$T+2$	.....	−13	−7	−9	.....	−38	−38	−12

* Base rate for each sample was determined during hour $(T-1)$ immediately prior to time (T) of addition of 2,4-D to substrate. Measurements of activities were made during first $(T+1)$ and second $(T+2)$ hours after treatment.

† Changes are expressed in relation to base rate $(T-1)$ of each sample valued as 100.

‡ Changes in rate for untreated seedlings, measured 1 and 2 hours after T, were applied as factors to base rates $(T-1)$ measured for treated samples to obtain values for latter samples which would be expected in the absence of 2,4-D during 1 and 2 hours after T. Differences between measured and expected rates for each sample are shown in relation to 100 assigned to expected rate for each sample at 1 or at 2 hours after T.

differences in volume size of samples might also be possible.

In the protocols (fig. 1) for control wheat (*W-C*) and mustard (*M-C*), lines *1* and *2* converge toward line *B* at the top. This indicates a progressive increase in rate of O_2 uptake during the test. Evidence of growth was not visible, and other growth data were not obtained. In other similar tests it was not possible to measure a significant increase in the weight of embryonic tissue of samples during such test periods. However, this

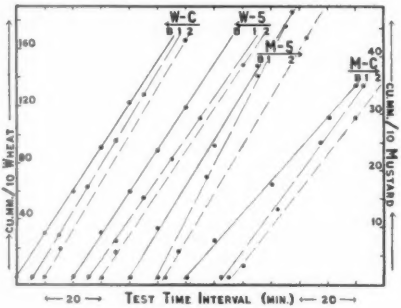

FIG. 1.—Protocols of tests of O_2 uptake by wheat (*W*) and mustard (*M*) seedlings: (*B*) untreated during twenty-fourth hour of development, (*C*) untreated and (*5*) treated with 5 p.p.m. of 2,4-D during twenty-fifth and twenty-sixth hours (*1* and *2*, respectively) of development. Treatments applied to seedlings 24 hours old.

relation for controls most likely is an expression of small, possibly unmeasurable growth which accompanied or resulted in the increase in O_2 uptake of the samples. Change in rate of O_2 uptake per unit weight of tissue possibly, but not necessarily, was involved.

Base rates of O_2 uptake were different for different samples (table 1, measured rates at *T-1*). It seems probable that the volumes of embryonic tissue would have varied somewhat among samples and that initial rate was proportional to sample volume. During the 2 hours of treatment, rates of O_2 uptake of treated samples did not increase as much as did those of untreated samples (table 1, change in rate measured). Protocol O_2 uptake lines (*1* and *2*) for seedlings in 5 p.p.m. of 2,4-D converged less toward the base rate lines (*B*) than they did for respective control samples during the first and second hours after treatment (fig. 1). This may indicate an effect of 2,4-D on growth (weight of sample), an effect on rate of O_2 uptake per unit weight of tissue, or a complex involving both effects.

A smoother relation between rate of O_2 uptake and concentration or duration of treatment with 2,4-D resulted when the data for measured rates of treated samples were somewhat adjusted to account for the probable variation in initial volume of the samples (table 1, difference between measured and expected rates). Such calculation shows that the rate of O_2 uptake of wheat and mustard seedlings had been reduced 7–38% during 2 hours following application of 1 p.p.m., or more, of 2,4-D. The response was proportional but not parallel to duration of treatment and was irregular in relation to concentration of 2,4-D.

In all instances after 2 or 18 hours of pretreatment with 2.5–10 p.p.m. of 2,4-D (table 2), the rates of O_2 uptake and of CO_2 evolution by intact wheat seedlings 30 or 48 hours old were reduced compared with controls. The decreases in rates of activity were somewhat proportional to concentration and to duration of treatment with 2,4-D. Slightly greater decrease in rate of O_2 uptake than in rate of CO_2 evolution occurred in seedlings 48 hours old, so that respiratory quotients (*RQ*) of the treated samples were slightly higher than those of controls.

During the test period of 18 hours the mean dry weight of untreated samples

increased 155%. Increase in dry weight of embryo of treated samples was 18–20% less than in controls. When evaluated on the basis of unit dry weight of embryo, decreases in activities resulting from treatment were always appreciably less and were more clearly and uniformly related to factors of concentration and duration of treatment than they were when calculated on the basis of seedling samples.

The *RQ* values, very close to 1.0 for untreated seedlings, suggest the functioning of an aerobic, hexose-type metabolism. Although treatments inhibited growth, the slightly higher *RQ* values for treated seedlings 48 hours old might be interpreted to indicate that anaerobic CO_2 evolution either was evoked or was not inhibited as much as aerobic CO_2 evolution.

Rates of CO_2 evolution and O_2 uptake by samples of mustard consisting of twenty-five seedlings 74–78 hours old, pretreated for 2–6 hours with 0.25–5 p.p.m. of 2,4-D, were in all cases lower than those for controls (table 3, fig. 2). As in wheat, the amount of reduction of an activity was generally proportional to concentration and duration of treatment with 2,4-D. Differences in initial samples may in part explain the fact that treated seedlings 78 hours old were heavier than controls.

In comparison with controls in this test, for all treatments the reduction of CO_2 evolution was as great as, and usually greater than, reduction of O_2 uptake. The *RQ* values of treated seedlings were lower than those of controls. The latter were higher than in other tests of mustard (table 6). Aerobic oxidation processes in treated seedlings may have been less nearly complete than in controls, or treatment may have altered the type of substrate utilized in such seedlings which

TABLE 2

MEAN RATES OF CO_2 EVOLUTION AND OF O_2 UPTAKE AND *RQ* VALUES FOR WHEAT SEEDLINGS 30 OR 48 HOURS OLD, TREATED FOR 2 OR 18 HOURS WITH 2,4-D. RELATIVE RATES SHOWN FOR DUPLICATE TREATED SAMPLES, 10 SEEDLINGS EACH, IN COMPARISON WITH VALUE OF 100 FOR MEAN RATE OF UNTREATED SAMPLES IN EACH TEST

Seedling age (hr.)	Pretreatment time (hr.)	2,4-D concentration (p.p.m.)	Rate of activity				*RQ*		Dry weight of embryo
			CO_2 (cu. mm./hr.)		O_2 (cu. mm./hr.)				
			Per 10 seedlings	Per mg. of embryo	Per 10 seedlings	Per mg. of embryo	Per 10 seedlings	Per mg. of embryo	
30	2	0	221.1	15.28	217.1	15.24	1.02	1.04	13.3±1.4 mg.*
		2.5	82	97	85	99	0.99	0.99	.....
		5	89	95	89	93	1.02	1.02	.....
		10	.....	.....	85	91	.....	.....	.....
48	18	0	384.9	11.06	394.2	11.42	0.98	0.97	34.0±1.2 mg.†
		2.5	70	93	69	89	0.98	1.01	82±6†
		5	68	85	66	82	1.01	1.01	78±4†
		10	57	82	54	77	1.02	1.02	71±8†

* Mean, and maximum variation per 14 replicates.
† Mean, and maximum variation per 3 replicates.

have relatively low carbohydrate reserves but are relatively high in fat. To the extent that the oxidative processes may be similar and may be similarly affected by treatment, the comparative responses of wheat and mustard seedlings suggest that anaerobic metabolism in mustard is potentially relatively weak. The influence of 2,4-D on such metabolism likely would not be very great.

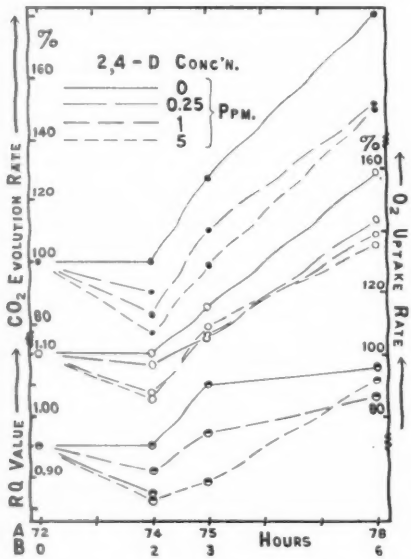

Fig. 2.—Mean relative rates of CO_2 evolution (*solid dots*) and O_2 uptake (*open dots*), and *RQ* values (*semisolid dots*) measured when duplicate twenty-five-seedling samples of mustard, 70 hours old, were treated with 0–5 p.p.m. of 2,4-D. Age of seedlings shown on line *A* and duration of treatment on line *B*.

Compared with controls, changes in the rates of CO_2 evolution and O_2 uptake and in the *RQ* values for mustard seedlings 26–48 hours old, pretreated for 2–24 hours with 2.5 or 10 p.p.m. of 2,4-D (table 4), were in general similar to those for older seedlings (table 3). Relative to the amount of decrease in rate of O_2 uptake resulting from treatment, the rates of CO_2 evolution and the *RQ* values for seedlings 32–48 hours old were reduced less than for seedlings 78 hours old. This could be considered partial evidence that anaerobic metabolism in the younger seedlings was potentially greater in amount or was less sensitive to 2,4-D than in the older seedlings.

When the data were treated to show cumulative change in the relative rates of activities (table 4), the distinct influence of treatment with 2,4-D in limiting the growth of seedlings, as well as in affecting their rates of metabolic activity, became more apparent. CO_2 evolution was only slightly nearer to that of controls than was O_2 uptake for the seedlings up to 32 hours old. The O_2 uptake of seedlings 48 hours old was appreciably more like that of controls than was CO_2 evolution. Altogether these data for mustard seem to give little clear indication of anaerobic metabolism greater or less sensitive to 2,4-D than aerobic metabolism in younger as compared with older seedlings.

On the basis of the tests described, and others, it appeared that 2,4-D caused marked effects which were expressed strongly within 3 hours following application of treatment. A number of tests were conducted in which seedlings of different ages (fig. 3) were pretreated for 3 hours immediately prior to measurement of their rates of gas exchange. Differences in the sizes and relative activities of seedlings of different types and ages made it desirable to use samples of different sizes in various tests. Data are summarized in terms of samples consisting of ten seedlings to facilitate comparison between activities of a type of seedling of any age in different tests. The data for wheat were also analyzed on the basis of unit dry weight of embryo (table 5, fig. 4).

TABLE 3

MEAN RATES OF CO_2 EVOLUTION AND OF O_2 UPTAKE AND *RQ* VALUES FOR MUSTARD SEEDLINGS 74 TO 78 HOURS OLD, TREATED FOR 2 TO 6 HOURS WITH 2,4-D. ACTIVITIES FOR DUPLICATE SAMPLES EXPRESSED ON BASIS OF 10 SEEDLINGS

Seedling age (hr.)	Pre-treatment time (hr.)	2,4-D concentration (p.p.m.)	CO_2 evolution		O_2 uptake		*RQ*	Mean dry weight (mg.)
			Cu. mm./hr.	%	Cu. mm./hr.	%		
74	2	0	128.2	100	135.3	100	0.95	24.0±1.5†
		0.25	113.1	90	131.3	97	0.87	
		1	106.5	83	117.0	87	0.91	
		5	99.3	77	115.1	85	0.86	
75	3	0	163.5	100	156.0	100	1.05	
		0.25	.....	.....	142.2	91	.....	
		1	142.9	87	147.4	94	0.97	
		5	127.5	78	143.0	92	0.89	
78	6	0	232.0	100	215.4	100	1.08	24.5‡
			9.09*	100*	8.49*	100*	1.07*	
		0.25	.....	.....	195.2	91	.....	26.2‡
					7.35*	87*		
		1	194.2	84	188.3	87	1.03	27.6‡
			7.11*	78*	6.88*	81*	1.03*	
		5	192.9	85	182.7	85	1.06	26.6‡
			6.86*	75*	6.57*	75*	1.04*	

* Calculated per mg. dry weight of embryo. † Twelve replicates. ‡ Three replicates.

TABLE 4

EFFECTS OF 2,4-D ON GAS-EXCHANGE RELATIONS OF MUSTARD SEEDLINGS. MEAN VALUES SHOWN FOR ACTIVITIES OF DUPLICATE SAMPLES SELECTED AT 24 HOURS AND THEN TREATED WITH 2,4-D FOR 2–24 HOURS PRIOR TO MEASUREMENT OF ACTIVITIES

Seedling age (hr.)	Treatment		Measured rate of activity		*RQ*	Relative rate of activity			
	Time (hr.)	Concentration (p.p.m.)	Cu. mm./hr. per 10 seedlings			Per seedling age*		Cumulative change in rate†	
			CO_2	O_2		CO_2	O_2	CO_2	O_2
26	2	0	34.8	40.7	0.86	100	100	0	0
		2.5	31.5	39.4	0.80	91	97	− 9	− 3
		10‡	29.3	37.0	0.79	84	91	− 16	− 9
27	3	0	.....	44.0	.....	.....	100	.....	+ 8
		10	.....	38.7	.....	.....	88	.....	− 5
32	8	0	51.9	59.1	0.87	100	100	+ 49	+ 46
		2.5	44.6	51.5	0.87	86	87	+ 28	+ 27
		10	41.2	46.6	0.88	80	79	+ 18	+ 15
48	24	0	74.1	98.2	0.75	100	100	+113	+142
		2.5	64.0	90.7	0.71	86	92	+ 84	+123
		10	55.6	78.7	0.71	75	80	+ 60	+ 94

* Activities of untreated seedlings of each age valued as 100.
† Activities of untreated seedlings 26 hours old valued as 100. ‡ Only one determination of O_2 uptake.

Progressive increase in the rates of O_2 uptake and CO_2 evolution per ten control seedlings clearly reflect the increase in size of wheat (table 5) and mustard (table 6) seedlings as they became older. The change in rates per milligram of wheat tissue showed that the fraction of the seedling having lower rates of activities increased with age. The *RQ* values for mustard clearly tended to decrease as the seedlings became older, likely indicating a progressive depletion of carbohydrate reserve and a shift toward a noncarbohydrate substrate for metabo-

FIG. 3.—Representative seedlings at time of treatment. *Upper* (*left to right*), wheat 18, 24, 30, and 50 hours old; *lower* (*left to right*), mustard 30, 50, and 70 hours old.

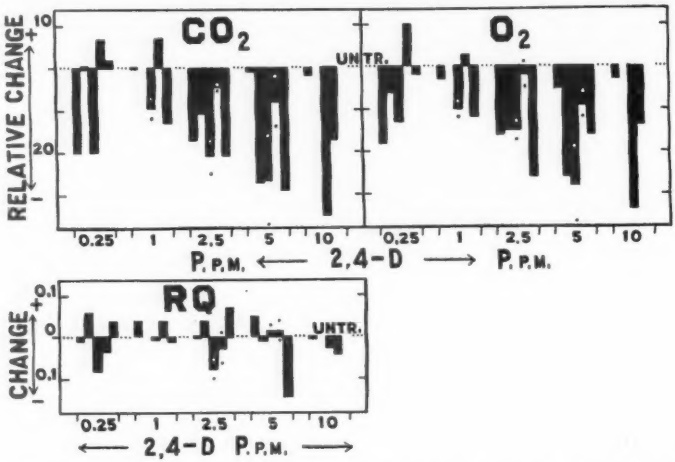

FIG. 4.—Mean relative changes in volume rates of CO_2 evolution and O_2 uptake and changes in *RQ* values for wheat seedlings 18–60 hours old after 3 hours under treatment with 0.25–10 p.p.m. of 2,4-D. For each 2,4-D concentration five positions are shown, representing (*left to right*) seedlings 18, 24, 30, 50, and 60 hours old. Data calculated on the basis of 1 mg. of dry weight of embryonic tissue. In all tests activities of untreated seedlings valued as 100 (0 on figure scale). Dots appearing in line vertically with a bar indicate maximum variations of means for activities observed in various tests involving age of seedling and treatment concerned. No tests made on seedlings 24 hours old with 1 or 10 p.p.m. or on seedlings 60 hours old with 10 p.p.m.

TABLE 5

MEAN RATES OF CO_2 EVOLUTION AND OF O_2 UPTAKE AND *RQ* VALUES FOR SAMPLES OF WHEAT SEEDLINGS 18–60 HOURS OLD TREATED FOR 3 HOURS WITH 0.25–10 P.P.M. OF 2,4-D

Seedling age (hr.)	Test no.	2,4-D concentration (p.p.m.)	Rate of activity of seedlings and of seedling tissue				*RQ*	
			CO_2 (cu. mm./hr.)		O_2 (cu. mm./hr.)			
			Per 10 seedlings	Per mg. of embryo	Per 10 seedlings	Per mg. of embryo	Per seedlings	Per embryo
18	1	0	81.6	21.11	79.7	21.19	1.02	0.99
		0.25	65.0	16.96	66.0	17.38	0.98	0.98
		2.5	77.0	17.60	75.3	17.70	1.02	0.99
	2*	0	76.5	15.51	73.1	14.82	1.05	1.05
		1	76.4	15.60	70.1	14.30	1.09	1.09
		5	76.0	15.40	69.3	14.04	1.10	1.10
		10	75.0	15.20	71.1	14.42	1.05	1.05
24	1†	0	144.2	17.36	146.5	17.73	0.98	0.98
		0.25	136.5	17.33	133.2	16.61	1.02	1.04
		2.5	120.3	15.41	118.3	15.05	1.02	1.02
		5	101.8	12.69	105.8	13.05	0.96	0.97
30	1	0	243.4	17.46	241.1	17.47	1.01	1.00
		0.25	197.7	14.03	211.5	15.33	0.93	0.92
		2.5	179.1	13.09	194.0	14.48	0.92	0.90
	2	0	173.2	16.42	177.9	16.73	0.97	0.98
		1	186.3	14.41	194.0	14.74	0.96	0.98
		5	149.1	10.28	156.4	10.16	0.95	1.01
	3	0	182.5	15.05	187.8	15.56	0.97	0.97
		2.5	134.9	12.45	147.8	13.51	0.91	0.92
	4	0	183.8	16.55	190.3	17.13	0.97	0.97
		1	200.1	15.36	195.2	15.84	1.03	0.97
		5	177.8	14.00	180.8	14.46	0.98	0.97
		10	165.8	10.72	166.4	11.32	1.00	0.95
50	1	0	302.2	12.25	323.5	13.33	0.93	0.92
		0.25	269.1	13.11	304.3	14.66	0.88	0.89
		2.5	235.0	11.66	278.1	13.49	0.85	0.86
	2	0	294.2	10.76	307.0	11.41	0.96	0.94
		1	333.4	11.51	336.8	11.70	0.99	0.98
		5	268.8	9.34	294.3	10.05	0.91	0.93
	3†	0	399.6	11.71	422.0	12.25	0.95	0.96
		2.5	379.1	11.22	393.9	11.57	0.96	0.97
		5	387.5	11.51	392.4	11.50	0.99	1.00
		10	348.7	9.78	377.5	10.59	0.92	0.92
60	1	0	406.7	12.30	447.0	13.38	0.91	0.92
		1	389.4	10.67	412.2	11.74	0.94	0.91
		5	285.9	8.71	379.0	11.24	0.76	0.78
	2	0	307.9	10.45	344.5	11.33	0.89	0.92
		0.25	376.1	10.71	402.5	11.14	0.93	0.96
		2.5	285.0	8.25	288.4	8.33	0.99	0.99

* Only one determination of O_2 uptake for all treatments in this test.

† Only one determination of CO_2 evolution for all treatments in this test.

lism. This shift, which was less evident for wheat seedlings, has been noted to be typical in previous studies of the metabolism of developing cereal seedlings (**4, 10**).

On an absolute basis, variation of activities of treated and untreated seedlings of given ages occurred among tests. Some part of this represented experimental error, but part of the variation must have resulted from qualitative and quantitative differences in detail of metabolic activity, which were developed in response to environment during culture of seedlings prior to their selection for treatment.

In only four instances—two at 30 hours, one at 50 hours, and one at 60 hours—did the rates (based on ten-seedling samples) of O_2 uptake and CO_2 evolution of wheat treated with 0.25–1 p.p.m. exceed the rate for untreated seedlings. These increases were from 7%

TABLE 6

MEAN RATES OF CO_2 EVOLUTION, O_2 UPTAKE, AND *RQ* VALUES FOR DUPLICATE SAMPLES OF MUSTARD SEEDLINGS TREATED FOR 3 HOURS WITH 0.25–10 P.P.M. OF 2,4-D. VOLUME RATE FOR UNTREATED SAMPLE VALUED AS 100 RELATIVE TO RATE FOR TREATED SAMPLES IN EACH TEST

Test no.	Seedling age (hr.)	Pretreatment time (hr.)	2,4-D treatment (p.p.m.)	Activity of untreated seedlings (cu. mm./hr./10 seedlings) and relative activity for treated seedlings		*RQ*
				CO_2	O_2	
33	30	3	0	40.2	42.6	0.94
			0.25	91	91	.94
			5	82	84	.92
35	30	3	0	33.3	39.2	.85
			1	97	91	.90
			10	86	89	.88
13*	50	3	0	93.5	136.8	.67
			2.5	89	94	.66
			10	87	90	.67
37	50	3	0	107.3	128.0	.84
			1	86	89	.80
			5	88	91	.81
34†	50	3	0	92.3	95.0	.97
			0.25	89	91	.94
			2.5	86	90	.93
			10	81	81	.96
15†	75	3	0	145.7	199.1	.73
			2.5	79	94	.62
			10	77	88	.64
14*	70	24	0	140.4	188.5	.75
			2.5	99	96	.78
			10	97	95	0.76

* Tests 13 and 14 were conducted as a series in which length of pretreatment time varied as indicated. CO_2 evolution rate was measured only on one sample in these tests.

† Three determinations of rate of O_2 uptake and one of CO_2 evolution made for each treatment in this test.

to 22% and were greater in the older than in the younger seedlings. The variation among rates for replicates in a test of older seedlings (8% or less when seedlings were 50 hours old or more) was usually greater than that (less than 5%) which occurred in tests of younger seedlings. When the data were calculated on the basis of unit weight of embryonic tissue, five of the apparent increases proved to be decreases, and the other increases were of lesser magnitude.

By far the main effect of 2,4-D was to reduce all rates of activity in proportion to the concentration applied. Some unexplainable variations from the smoothness of this general trend appeared for both wheat and mustard seedlings. The main difference between the responses of the two kinds of treated seedlings concerned the degree of changes in rates of O_2 uptake or of CO_2 evolution. The O_2 uptake of wheat was generally decreased by 2,4-D relatively more than that of mustard. This difference was irregular and not great.

The rate of CO_2 evolution of mustard was decreased in degree approximately equal to, or greater than, the reduction in the rate of O_2 uptake (table 6). Decreases in the rates of CO_2 evolution of wheat which occurred when low concentrations (0.25–2.5 p.p.m.) were applied to seedlings of different ages in several of the tests were relatively slightly less than the decreases in rates of O_2 uptake (table 5). In general, as a result of these responses the *RQ* values for treated mustard usually tended to remain similar to those of controls, or to decrease, while the *RQ* values for treated wheat increased slightly in several instances, or were similar to those of untreated seedlings.

For both kinds of seedlings of all ages, treatment with 5–10 p.p.m. of 2,4-D caused decreases in the rate of CO_2 evolution approximately equal to, or greater than, the marked decreases in the rate of O_2 uptake. The *RQ* values often were lower than controls. Evidence has been presented (**12, 18**) that anaerobic metabolism is normally stronger during the period of early germination than during the later development of some species. The results of all the tests on wheat and mustard suggest stronger anaerobic metabolism in wheat than in mustard. Though responses were sometimes irregular, a comparison of the complexes of responses in younger and older seedlings, especially wheat, seems to indicate somewhat stronger anaerobic metabolism during the earlier stages of development.

Discussion

Soon after several different chemical growth-regulators, in particular 2,4-dichlorophenoxyacetic acid (**7, 13, 21, 26**), are sprayed on vegetative tops, responses in parts of several species of dicotyledonous plants appear to involve mobilization of reserve carbohydrate, increase in content of soluble sugars, and increase in metabolic activity, often followed by decrease in soluble sugars and by the development of malconditions or by the death of the plants (**2, 13, 15, 22**). Hydrolyses of reserves and their mobilization over relatively short distances from seed-storage organs to the rapidly growing and metabolizing embryonic parts normally occur at high rates in seedlings (**1, 4, 10**). Germinating seedlings contain appreciable amounts of soluble sugars (**1**).

Many kinds of seedlings have been shown to be relatively highly sensitive to treatment with 2,4-D (**3, 8, 16, 25**). Development of malconditions, often death, frequently has been shown to follow soon after treatment of seedlings with relatively low concentrations of the acid. Al-

though growth was somewhat inhibited, O_2 uptake by wheat seedlings was stimulated by low concentrations of indoleacetic acid (**19**). This acid stimulated O_2 uptake and protoplasmic streaming in oat coleoptiles when applied in concentrations of 10 p.p.m. or less (**5, 23**).

There was very little, if any, distinct indication of any increase in metabolism of energy liberation in seedlings after treatment with 0.25–10 p.p.m. of 2,4-D. Usually such activity decreased soon after treatment. Results of tests of oxidative processes in seedlings and of tests on established plants (**7, 21**) would seem to indicate another instance of marked difference in response of tissues to treatment with 2,4-D.

In so far as judgment can be based on evidence from gas-exchange studies, the results of tests on wheat and mustard seedlings indicated that metabolism was decreased by treatment with 0.25–10 p.p.m. of 2,4-D. In general, the apparent responses of the monocotyledonous and dicotyledonous species appeared similar, even though reports of investigations on seedlings have clearly shown a differential herbicidal effect of 2,4-D on various plant groups and varieties (**3, 8, 16, 25**).

At least in the case of wheat and mustard, the differential herbicidal effect does not seem to be primarily concerned with a distinct difference in response of the energy metabolism of the seedlings. Nor does it appear likely, on the basis of gas-exchange responses, that abnormal, rapid depletion of metabolic and growth substrate, induced by treatment with 2,4-D, would be a major factor possibly contributing toward development of starvation as a part of the complex of malconditions reported to occur in seedlings after treatment.

HSUEH and LOU (**9**) recently have reported that metabolic activity and germination of seedlings were distinctly decreased and delayed, or completely inhibited, during tests lasting 6 days when seeds of a number of species were treated with relatively high concentrations (up to 1000 p.p.m.) of 2,4-D. In general, the gas-exchange responses which they observed for treated seed, especially barley, seemed qualitatively similar to those which are here described for wheat and mustard seedlings treated with 10 p.p.m., or less, of the acid. They found that barley, wheat, and seed of a number of dicotyledonous species did not germinate when treated. Rice seed showed germination activity of some nature which was somewhat delayed compared with that of untreated seeds. Mainly by means of manometric tests they found that apparent anaerobic oxidation in rice was engendered by 2,4-D. This response seemed related to germination activity in rice. The tenable, but not entirely proved, suggestion was made that the anaerobic oxidation processes in rice treated with 2,4-D were able to supply energy required for endothermic processes of germination.

In considering the differences observed in the relative amounts of germination of rice compared with other seeds tested by HSUEH and LOU, it is interesting to note the relative rates of respiration. The apparent aerobic activity of rice after treatment was greater than that of other similarly treated seedlings. It seems possible that the greater development by treated rice seedlings could as well be correlated with their higher level of aerobic respiration.

The difficulty one experiences in following treatment by HSUEH and LOU of their data precludes the possibility of any

direct comparison between results of their manometric tests and those of others. Moreover, their manometric data are difficult to evaluate critically because quantitative expression of volume rates of gas exchange or the constants to allow calculation of their data in terms of volume rates were not given. It did not appear that the progressive development of the effect of treatment on growth (volume and uniformity of samples) was given detailed consideration in the evaluation of the results of their manometric tests.

In some tests conducted by the writer in an atmosphere of 100% nitrogen, the rate of CO_2 evolution by rice seedlings was slightly greater in 2.5 p.p.m. of 2,4-D than for controls during a period of approximately 6 hours after initiation of treatment of seedlings 30 hours old. The rate for seedlings treated with 2,4-D then decreased and was distinctly less than that of anaerobic controls before the twenty-fourth hour of treatment.

In somewhat the same manner as do anaerobic environments or treatments with oxidase inhibitors (**1, 12, 24**), treatment with 2,4-D does appear to elicit the expression of anaerobic activity in treated tissues in which potentials for such activity exist. Part of the results of reported studies on the influence of 2,4-D on the metabolism of energy release can be interpreted to indicate this effect in some degree.

Summary

1. Seedlings of wheat and mustard were selected when 18–24, or more, hours old and were treated with 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations up to 10 p.p.m. Manometric measurements of the rates of CO_2 evolution and of O_2 uptake were made at various intervals from the first through the twenty-fourth hour after treatments were initiated. A number of tests were conducted in which the activities of seedlings 18–70 hours old were measured after these had been exposed for 3 hours to 2,4-D treatments.

2. The main responses were reduction in rates of CO_2 evolution and of O_2 uptake by both species at all ages. Apparent reduction of activity, generally in proportion to concentration of 2,4-D, was measured during the first hour after initiation of treatment, and such reduction increased with duration of treatment.

3. In a number of tests, especially on wheat the rate of CO_2 evolution was decreased less than the rate of O_2 uptake, so that *RQ* values of treated seedlings were slightly higher than those of controls. These responses, which appeared more irregularly and less distinctly for mustard than for wheat and which appeared somewhat more frequently in earlier stages of development of seedlings, are discussed in relation to the herbicidal effect of treatment with 2,4-D.

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EFFECTS OF SEVERAL SULFA- COMPOUNDS ON NUCLEAR AND CELL DIVISION[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 593

THOMAS C. FULLER

Introduction

Since the discovery that polyploidy can be produced in plants almost at the will of the investigator through the use of colchicine (**2, 3, 4**), considerable interest has developed in the possibilities thus opened up, and experiments with other chemicals have been going on. More recently EIGSTI (**5**) reported that "mitotic irregularities" were induced in the pollen tubes of *Tradescantia occidentalis* when they were treated with dilute concentrations of sulfanilamide (1:10,000). Greater concentrations inhibited the formation of the tube. TRAUB (**9**) found polyploid ($4n$) and numerous binucleate cells in *Allium* roots after 48 hours of treatment with sulfanilamide (0.5%). PETERS (**8**) reported that it produced an extreme contraction of the chromosomes of *Allium* at metaphase and inhibited the spindle mechanism. He noted that after reaching the metaphase stage, the chromosomes reverted to an active metabolic condition without completing the mitotic cycle. The reversions occurred both before and after the division of the centromeres, resulting in the production of tetraploid nuclei which were observed in blocked metaphases as early as 8 hours after treatment. He also found that sulfanilamide inhibited the entrance of cells into mitosis and that divisions rarely occurred after 48 hours of treatment. Although less effective than colchicine in inducing polyploidy in *Allium* (**8**), sulfanilamide has been more effective in some species of *Vaccinium* (**6**).

These reports led to a reinvestigation of the effects of sulfanilamide on nuclear and cell divisions, together with the effects of several other sulfa- compounds: viz., sulfadiazine, sulfaguanidine, sulfamerazine, sulfapyridine, and sulfathiazole.

Material and methods

The bases of bulbs of *Allium cepa* L. were placed in tap water in Stender jars in the laboratory for a period of 24–48 hours until roots averaging approximately an inch in length were developed. These roots, while still attached to the bulbs, were treated by immersing them in aqueous solutions of the sulfa- compounds.

Preliminary tests of the effects of sulfanilamide were made in solutions of 0.125, 0.25, and 0.5% concentration by weight in tap water at 20° C. Because the greatest effects occurred in the 0.5% solution, the weaker concentrations were not used in the later experiments. All the other sulfa- compounds were used at 0.1% concentration by weight in tap water because of their low solubility. Even this quantity was slightly above the saturation point of these compounds in water, and a slight excess of solid material was present in each solution.

Treatments were started at 12:30 P.M., and the solutions were changed every 24 hours. Root tips were collected

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at intervals up to 96 hours following the start of the treatments. After 48 hours some treated roots were returned to tap water, and collections were made from them 24 and 48 hours later. A few treated roots were also returned to tap water at the end of 96 hours, but, since they showed essentially the same responses as those returned to tap water after 48 hours of treatment, this procedure was not repeated in later experiments. Control bulbs were continued in tap water, which was also changed at 24-hour intervals. Both treated and control root tips were fixed in Navashin's solution, handled according to the tertiary-butyl-alcohol method, and imbedded in paraffin. Sections cut transversely at 20 μ were stained with Heidenhain's iron-alum hematoxylin with a counterstain of Orange G in clove oil.

Results

The first observable response following the immersion of the roots in the solutions of the sulfa- compounds was the cessation of visible elongation, and it was not renewed during the 96-hour period of treatment. The roots also became progressively discolored with a brownish tinge during treatment.

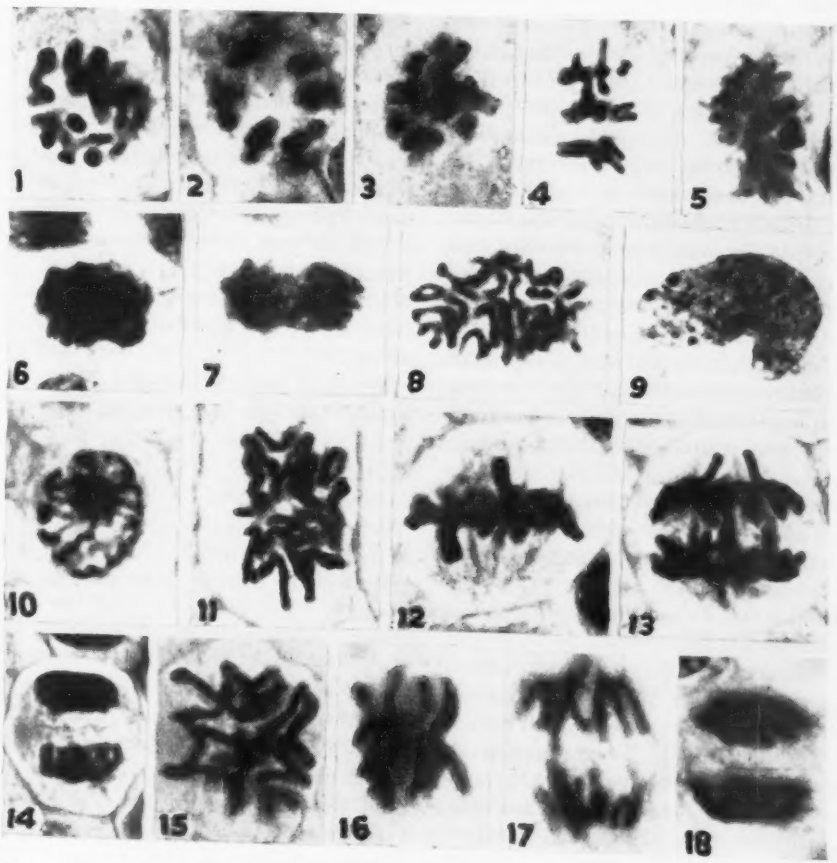
Approximately half the roots immersed in 0.5% sulfanilamide showed an increase in diameter in the morphological region of elongation within 24 hours. The enlargements reached a maximum size within 48 hours after treatment as a result primarily of an increase in size of the cortical cells. Similar tumors were not formed following treatment with any of the other sulfa- compounds except for one bulb in which an initial swelling was noticed in approximately half the roots after 72 hours of immersion in the sulfapyridine solution. These tumors, comparable to those from the sulfanilamide treatments, reached a maximum size at the end of 96 hours. On being returned to tap water, all the treated roots, including those showing tumors, resumed growth. The new growth was noticeably whiter than the darkened older portion.

In a series of preliminary experiments with sulfanilamide at 0.125, 0.25, and 0.5% concentration in tap water at 20° C., the elongation of roots in the two lower concentrations was inhibited, but tumor formation did not occur. Cells from these roots showed late prophase and metaphase stages with slightly shortened chromosomes and a much slower rate of division than in control roots. At 0.5% concentration sulfanilamide had a more pronounced effect on mitosis. Preliminary examinations of roots at hourly intervals up to 10 hours following the start of treatment showed a progressive shortening of the chromosomes at the late prophase and metaphase stages up to 3 hours, when the maximum contraction appeared to be reached. Because the changes occurred very slowly, later examinations were made at 6, 12, 24, 48, 72, and 96 hours after immersion in the solution.

Mitosis was found to occur in a few cells throughout the 96-hour period of treatment but at such a slow rate and in so few cells that no visible elongation of the treated roots took place during this period. The prophase nuclei responded to sulfanilamide by developing unusually contracted and condensed chromatids. In early prophases the chromosomes were scattered throughout the nucleus. Spiralization of the chromatids could rarely be observed, since it was somewhat obscured by condensed, densely stained material. While still in the mid-prophase stage, the chromosomes were markedly contracted (fig. 1). In later prophases they were scattered around

the inner periphery of the nuclear membrane, the center of the nucleus being clear (fig. 2). In one series of treatments no prophase stages were observable at 48 hours. This occurred under the same experimental conditions as in the other treatments but was not observed again.

The metaphase figures containing the much shortened chromosomes appeared flat when compared with the same stages in the controls (figs. 3, 15). The chromatids were characteristically parallel to each other throughout the metaphase and remained thus until disruption of the



FIGS. 1–18.—Figs. 1–9, cells after treatment with 0.5% sulfanilamide: fig. 1, prophase, 12 hours; fig. 2, late prophase with shortened chromosomes, 12 hours; fig. 3, metaphase with shortened chromosomes, 12 hours; fig. 4, metaphase with disrupted spindle, 48 hours; fig. 5, early reversion stage, metaphase, 96 hours; fig. 6, reversion stage, metaphase, 48 hours; fig. 7, reversion stage, anaphase, 48 hours; fig. 8, metaphase, tetraploid, 96 hours; fig. 9, lobed nucleus from cortical cell of tumor, 96 hours. Figs. 10–14, cells treated with other sulfa- compounds: fig. 10, prophase, sulfathiazole, 12 hours; fig. 11, metaphase plate, sulfadiazine, 6 hours; fig. 12, metaphase, side view, sulfapyridine, 96 hours; fig. 13, anaphase, sulfapyridine, 96 hours; fig. 14, telophase, sulfamerazine, 48 hours. Figs. 15–18, controls, cells untreated: fig. 15, metaphase plate; fig. 16, metaphase, side view; fig. 17, anaphase; fig. 18, telophase.

spindle mechanism occurred. This breakdown first became evident when the metaphase figure was no longer flattened. Concurrently, the chromatids were repelling each other strongly even before the division of the centromeres (fig. 4). The centromeres appeared to divide, however, before the chromosomes reverted to the metabolic condition. Reversion stages were found in material at 48–96 hours (figs. 5, 6). A number of anaphases in which the spindle mechanism had disrupted and in which the chromosomes were reverting to the metabolic state without completion of the mitotic cycle were found at 48 hours (fig. 7). These cells may possibly have been in a late metaphase or anaphase condition at the beginning of treatment. Binucleate cells resulting from the failure of cell-plate formation were also present; probably these cells had been in a late anaphase or telophase stage when treatment was started, and the consequent breakdown of the spindle mechanism resulted in their development. Metaphase and anaphase figures with the tetraploid number of chromosomes were found 96 hours following the application of sulfanilamide (fig. 8), but no other polyploidy was found.

The greatly enlarged cortical cells of the tumors had a large central vacuole, but their nuclei appeared similar to those of the controls. A few nuclei, in the metabolic condition, were much larger and were possibly tetraploid. A few of the greatly enlarged cells showed lobed nuclei resembling those of certain secretory cells or cells of maturing xylem vessels (fig. 9).

After immersion in 0.5% concentration of sulfanilamide for 48 hours, some bulbs of each series were transferred to tap water. No tetraploidy was found in cells from these roots at 24 and 48 hours after being returned to tap water.

Roots treated with the other sulfa-compounds—sulfadiazine, sulfaguanidine, sulfathiazole, sulfamerazine, and sulfapyridine—all showed the same general responses. Although there was no visible elongation following the application of the compounds, all stages in the mitotic cycle were found at all intervals during treatments, but with the division rate markedly retarded. The prophase and telophase stages appeared similar to those in controls. The metaphase and anaphase figures showed shortened chromosomes, but the degree of contraction was less than that resulting from treatment with 0.5% sulfanilamide, although similar to that of chromosomes in cells treated with the weaker concentrations of sulfanilamide. The principal effect was the marked slowing of the rate of divisions.

Roots of the one bulb that produced tumors in the sulfapyridine solution failed to show the cytological effects resulting from 0.5% sulfanilamide, except for the enlargement of the cortical cells. The mitotic figures were similar to those in roots from the other bulbs treated with sulfapyridine which developed no tumors.

Discussion

The results of this investigation confirmed the previous reports (**8, 9**) of the gross effects of sulfanilamide in that elongation of the roots ceased and tumors were developed in the morphological region of elongation. In the present experiments tumors were never formed on more than half the roots of any bulb. No visible differences could be detected between the nuclei of roots that formed tumors and those that did not, whether treated or untreated, except that an occasional nucleus in the tumor showed a lobed outline. Tumor formation did not occur following treatment with the other

sulfa- compounds, except in one bulb treated with sulfapyridine. Enlargement of the cortical cells to form tumors was therefore probably a result of a physiological disturbance in these cells.

All the sulfa- compounds prevented visible elongation of roots, while those of the controls grew to the bottom of the containers during the same time interval. Mitosis occurred in some cells of all the treated roots, except for one series of treatments with sulfanilamide in which mitosis was completely blocked at 48 hours. This agrees with the results of PETERS (8), although complete blocking of mitosis was, however, not again observed in any of the four repetitions with sulfanilamide.

The results with 0.5% concentrations of sulfanilamide were generally the same as those found by PETERS except that the responses were slower than he reported. Reversion stages were found at 48–96 hours after treatment began. PETERS reported them as early as 3 hours after treatment, with blocked metaphases in tetraploid nuclei as early as 8 hours. Only after 96 hours of treatment were tetraploid nuclei observed in the present experiments. In some of these the chromosomes appeared in flattened metaphase plates, although others showed apparently normal anaphase configurations during the time in which the roots were still immersed in the sulfanilamide solution.

According to PETERS, the prophase stages in treated roots were similar to those of the controls, with the chromatids a little more distinct. In the writer's material the chromatids appeared somewhat obscured by a slight cloudiness around them during the early prophase stages and were therefore less distinct than in the controls. In later prophase stages, however, the chromatids were more distinct than in the controls. During the late prophase stages the chromosomes may be as much shortened as they become in any of the metaphase stages. These latter stages were similar to those resulting from colchicine treatment, according to figure 4 of BERGER and WITKUS (1) except that this figure showed no nuclear membrane. They interpreted this as representing a disruption of the spindle mechanism at metaphase, with the chromosomes grouped around an achromatic sphere. PETERS reported that, following such a grouping, reversions to an active metabolic condition occasionally occurred. In the present experiments all the chromosomes appeared to reach the metaphase-plate stage before there was any indication of the disruption of the spindle. It seems probable that a breakdown of the nuclear membrane in a late prophase, when the short highly condensed chromosomes are arranged peripherally around the clear center of the nucleus, would result in the appearance described. Thus, what PETERS and BERGER and WITKUS interpreted as a type of disruption of the metaphase may instead be a late prophase in which the nuclear membrane had been dissolved and in which the chromosomes were reverting to an active metabolic condition without first being arranged in a metaphase plate.

The marked differences in results of treatment with sulfanilamide (0.5% concentration) and of those with the five other sulfa- compounds (0.1% concentrations) were possibly correlated with the lower solubility in water of the latter. This conclusion would be in accord with the findings of ÖSTERGREN and LEVAN (7), who used benzene, cyclohexane, and thiophene and various halogen derivatives of these compounds—monobromobenzene, monochlorobenzene, bromocyclohexane, dibromothiophene, etc.

No explanation can at present be offered for the more rapid results obtained by PETERS with 0.5% solutions of sulfanilamide than the consistently slower effects observed during the course of this investigation.

For demonstrating mitosis to the beginning student it is suggested that onion roots be immersed for 6 hours, before being fixed, in a 0.1% concentration of one of the sulfa- compounds. Under these conditions all stages of the mitotic cycle are essentially normal except that the chromosomes are thicker and shorter and therefore less entangled. Flat metaphase figures are obtained in which the sixteen chromosomes are clearly visible and in which the chromatids also show more clearly in the late prophases than in untreated material.

Summary

1. Onion roots approximately 1 inch long were immersed in solutions of 0.5% sulfanilamide and of 0.1% in tap water of the following sulfa- compounds: sulfadiazine, sulfaguanidine, sulfamerazine, sulfapyridine, and sulfathiazole. These solutions were at or slightly above the maximum solubility of these compounds in water at 20° C. The solutions were changed every 24 hours, as were the controls in tap water. Fixations of the roots were made after 6, 12, 24, 48, 72, and 96 hours of treatment. Some bulbs from each series were returned to tap water after 48 hours and others after 96 hours.

2. There was immediate cessation of visible root elongation in all the treatments, but approximately half the roots of the bulbs treated with sulfanilamide developed tumors within 48 hours, owing to enlargement of the cortical cells in the region of elongation. In only one other treatment were such tumors formed—on about half the roots of one bulb immersed in sulfapyridine.

3. In roots treated with sulfanilamide normal-appearing mitosis continued in a small number of the cells of the cortex and apical meristem throughout the 96-hours period of treatment. The duration of the mitotic process seemed to be materially lengthened. Disruption of the spindle mechanism and delay in the separation of the centromeres of the chromosomes were observed. As a result of the inhibition of the spindle mechanism, reversion stages occurred which resulted in the return of the anaphase chromosomes to the metabolic condition without completion of the mitotic cycle and in the production of tetraploid metaphase and anaphase figures within 96 hours after application of sulfanilamide. In no case, however, was a tetraploid cell found in division after normal growth had been resumed in tap water. Binucleate cells, resulting from the failure of the formation of the cell plate, were observed after 48 hours of treatment. The nuclei of the cortical cells in the enlarged regions appeared normal in most cells, although some nuclei were much larger than others and than those of the controls. These large nuclei were probably tetraploid. A few other nuclei showed irregular, lobed outlines. No micronuclei were found, in contrast to the report of PETERS. The only polyploidy observed was tetraploidy.

4. Mitosis was completely blocked at 48 hours in one series of treatments with sulfanilamide. In these roots no mitotic activity and no characteristic tumor formation occurred.

5. The other five sulfa- compounds induced essentially the same responses in all the roots. Although there was no visible elongation, mitosis nevertheless continued at a slow rate throughout the 96 hours of treatment. Even though the chromosomes were shorter than in the controls, the degree of contraction was

not so great as had occurred in cells treated with sulfanilamide. On return to tap water all treated roots resumed normal growth.

6. The markedly different responses to sulfanilamide and to the other sulfa-compounds occur possibly because of the greater solubility in water of sulfanilamide than of the others.

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FORMATIVE EFFECTS OF CERTAIN SUBSTITUTED CHLOROPHENOXY COMPOUNDS ON BEAN LEAVES[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY **594**

DANIEL F. BURTON

Introduction

One of the most striking effects of the application of substituted phenoxy compounds to plants is the induction of leaf modifications. Zimmerman (**10, 11**) has stressed the importance of the study of modifications in form induced by the hormone-like substituted phenoxy compounds by pointing out that protoplasm is capable of a variety of reactions and that it is desirable to investigate any mechanism that determines which of the potential reactions actually shall occur. He also suggested that, since form is determined by cell behavior, the substituted phenoxy compounds and the substances secreted by the nucleus may control cytoplasmic activity in a similar fashion and hence control form.

Foster (**5**) has indicated the paucity of data on leaf histogenesis compared with analogous data on stems and roots. Therefore, in the present experiments the normal growth pattern in leaves of the Red Kidney bean was examined as well

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as the growth patterns induced by the application of growth-regulating substances.

Material and methods

Red Kidney beans were planted in flats containing fertile loam soil and placed on greenhouse benches for germination. When the primary heart-shaped leaves were approaching their mature size and the first trifoliate leaves were starting to emerge from the bud, the flats were divided into four groups. The plants in the first group were treated with 0.5% 2-chlorophenoxyacetic acid in Carbowax 1500 applied to the upper surface of the blade near the bases of the primary leaves, in amounts estimated to contain 40 mg. of the mixture per plant, or 200 γ of the acid (3). The second, third, and fourth groups were treated with 4-chlorophenoxyacetic acid in lanolin, 2,4-dichlorophenoxyacetic acid (2,4-D) in lanolin, and 2,4-dichlorophenoxyacetic acid in Carbowax, respectively, in the same concentrations and in amounts approximating those used in the first group. Some plants in each flat were left untreated as controls.

Collections of buds, young leaves, and fully developed leaves were made from treated plants and controls. The material was fixed in Navashin's solution, handled according to the tertiary butyl–alcohol method, sectioned at 10–15 μ, and stained with KRAUS's modification of Fleming's triple stain.

Three sets of beans were planted at Chicago. The first set was planted on March 27, 1946, treated on April 6, and leaves and buds were collected on May 9. The second set was planted on April 11 and treated on April 22. At the time of treatment of this set, temperatures in the greenhouse were unseasonably high, and so many of the plants died that no collections were made. A third set was planted on May 21, treated on June 1, and collections were made on June 22. A fourth set, planted at State College, Mississippi, on March 26, 1947, was treated only with 2,4-D in lanolin and in Carbowax, on April 8; collections were made on April 29.

Observations

DEVELOPMENT AND APPEARANCE OF NORMAL TRIFOLIATE LEAVES.—The internal tissues of the lamina of a normal leaflet originate by the activity of subepidermal marginal meristems which are found on the lateral edges of the embryonic midrib nearer the adaxial surface (fig. 2*A*). Divisions of cells of these meristems give rise to four layers of cells between the upper and lower epidermis (fig. 2*B*). These layers plus the upper and lower epidermis constitute the plate meristem. The cells of the adaxial layer of internal tissue are larger than those of the other internal layers. Since the six cell layers in the lamina of the embryonic leaflet are still present in the lamina of a leaflet just prior to the development of intercellular spaces, all division of cells in the plate meristem, except divisions involving veins, must produce daughter cells in the plane of the plate meristem.

Initial cell divisions in the development of veins of the minor vascular network produce daughter cells vertical to the plane of the plate meristem. Such divisions occur in a single row of cells in the layer immediately beneath the layer of enlarged cells. In cross section, this row appears as a single cell. Figure 2*C*, illustrating a section cut obliquely to the row, shows a cell in which one such division has occurred, giving rise to the first two cells of the veinlet. To the right of these cells there were two divisions, producing three cells. Figure 2*D* shows a later stage

FIG. 1.—Trifoliate leaves from control (*A*) and treated plants (*B–H*). Plants treated with (*B*) 2,4-D in Carbowax 1500; reduced size and increased prominence of veins compared with control; (*C*) 2,4-D in lanolin; effects similar to *B*; (*D*) 2-chlorophenoxyacetic acid; leaflets reduced and prominent veins similar to *C*; (*E*) 4-chlorophenoxyacetic acid; narrow straplike leaflets ultimately produced; (*F, G, H*), 2,4-D in lanolin; leaves taken in succession to illustrate limitation in size of later leaves.

in the formation of smaller veins. An adjacent cell of the same layer has divided, as have several cells in the next lower layer.

A section through the same leaflet as that shown in figures 2*A* and 2*B* is illustrated in figure 2*E*. Although the tissues at the level of figures 2*A* and 2*B* were embryonic, those at the tip of the leaflet were mature, thus demonstrating that maturation begins at the tip. When fully expanded, the leaflet has a single layer of palisade parenchyma beneath the upper epidermis (fig. 3*A*), derived from the upper internal layer of enlarged cells illustrated in figures 2*A*, *B*, *C*, and *D*. The spongy mesophyll is derived from the other three internal layers (fig. 3*A*).

The vascular elements of the midrib (fig. 3*A*) and the largest veins are sur-

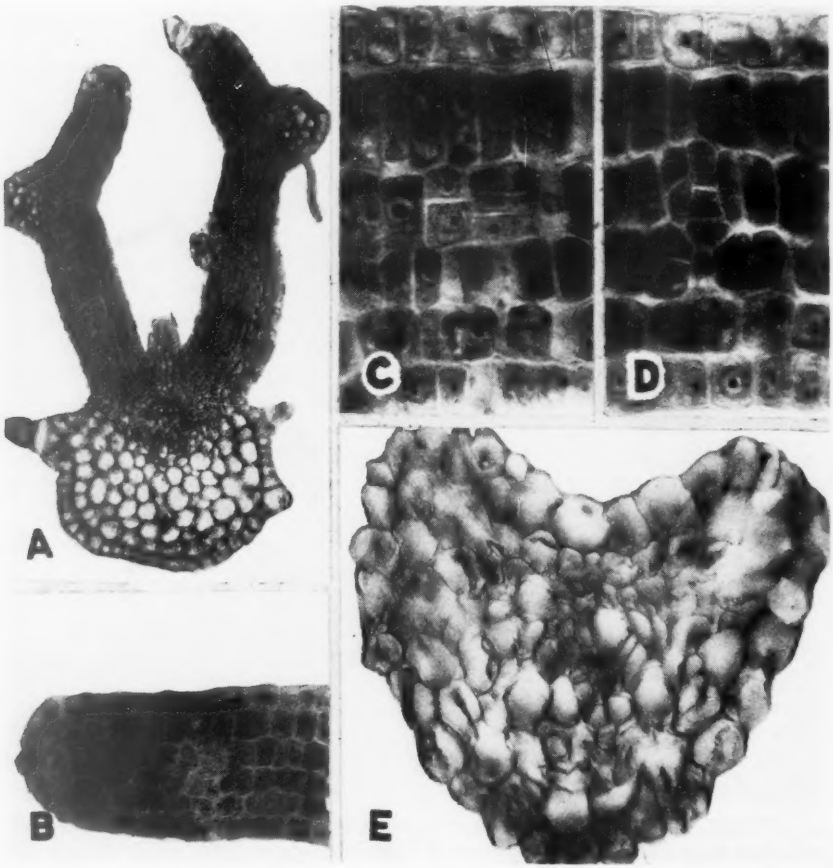

FIG. 2.—Embryonic leaflet of control. *A*, cross section through leaflet; lamina arising from embryonic midrib. *B*, detail of marginal and plate meristems shown in *A*. *C*, oblique section through row of cells initiating a vein in upper layer of spongy-mesophyll plate-meristem. *D*, cross section through similar row of cells, illustrating more advanced stage of vein formation. *E*, tip of leaflet, showing maturity of tissues at this region of same leaflet shown in *A* and *B*.

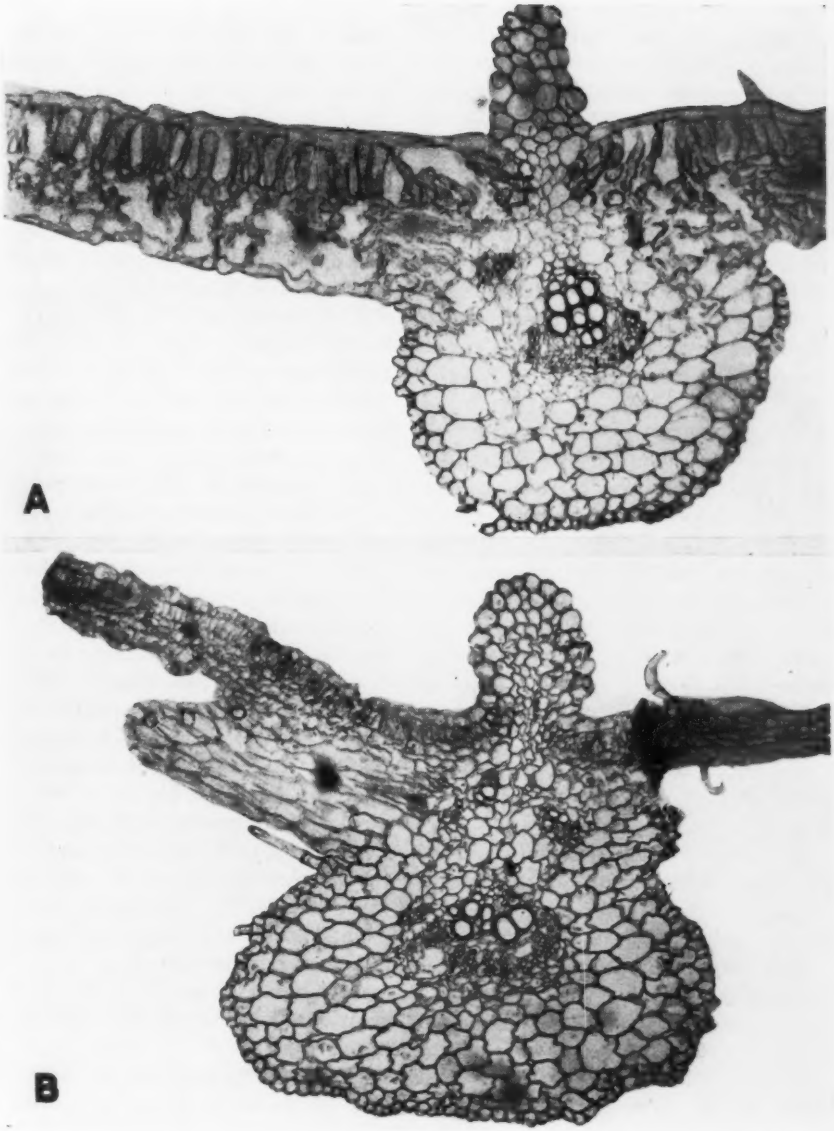

FIG. 3.—Cross section through mature leaflet in region of midrib of: (*A*) control with well-developed intercellular spaces in mesophyll and extensive region of parenchyma around vascular elements; (*B*) plant treated with 2-chlorophenoxyacetic acid; normal vascular tissue but no intercellular spaces in mesophyll.

rounded by an extensive parenchymatous bundle sheath which contains no chloroplasts. Smaller veins have a less extensive sheath, and in the smallest veins the sheath is only a single layer of parenchymatous cells often containing chloroplasts.

The mature leaf is trifoliate. The lamina of a leaflet is marked by a network of fine anastamosing veins (fig. 1*A*). A stipule subtends each of the lateral leaflets, with an additional pair beneath the terminal leaflet. Stipules persisted in the normal position in treated plants.

GROSS EFFECTS OF TREATMENTS.—Little or no epinasty or stem curvature occurred in plants treated with 2-chlorophenoxyacetic acid. At first the growth of these plants was comparable with that of controls, and the first few trifoliate leaves were essentially normal. Leaves produced later were smaller and had darker green leaflets with light-colored veins which, though less numerous, were more prominent than the veins of controls (fig. 1*D*).

The plants to which 4-chlorophenoxyacetic acid was applied showed pronounced epinasty and stem curvature in a few hours. Curvature frequently turned the stem through 180°, so that it appeared to have reversed from negative to positive geotropism. In the second lot, treated during a period of high temperatures, 80% of the plants were dead in 17 days. If a plant was not killed, its tip eventually resumed an upright growth pattern; by this return to the normal orientation an S-curve was produced in the hypocotyl and first internode. The first trifoliate leaf produced on a partly recovered stem was similar in appearance to the modified leaves produced on plants treated with 2-chlorophenoxyacetic acid. This leaf was just starting to emerge from the bud at the time of treatment. Similar but smaller leaves were sometimes developed subsequently, but most leaves formed later were narrow and straplike (fig. 1*E*). In such leaves the unpigmented midrib was wider than that of normal leaflets and often composed four-fifths of the total width of such anomalous structures. No other venation was apparent. The margins of the straplike leaflets were rolled and green.

Until leaves began to develop, the behavior of plants treated with 2,4-D, in either carrier, was similar to that of plants treated with 4-chlorophenoxyacetic acid. Epinasty and stem curvature occurred more rapidly and sometimes more severely in plants treated with 2,4-D than with either of the other two acids. All plants in the second lot to which 2,4-D had been applied in lanolin died within 17 days. When Carbowax was the carrier, most of the plants were alive after 17 days, but there were so few signs of recovery that all were discarded.

In those lots in which plants treated with 2,4-D showed partial recovery, the first few leaves produced resembled the modified leaves on plants treated with 2-chlorophenoxyacetic acid (figs. 1*B*, *C*). Leaves produced later were even smaller than earlier leaves on the same plant (figs. 1*F*, *G*, *H*). The most extremely modified leaves had midribs and veins fasciated into a wide band of unpigmented tissue, although the margins and occasional islets in the fasciated bands were green.

HISTOLOGICAL RESPONSES.—The modified leaves of plants treated with 2-chlorophenoxyacetic acid developed much like those of controls until the stage at which intercellular spaces normally would have appeared. Expansion of chlorenchymatous layers then ceased, and no intercellular spaces were formed

in either the palisade or spongy mesophyll (fig. 4*A*). Since the cells of these tissues contained chloroplasts, there was a greater amount of green pigment in a given volume of the leaflet than in a like volume of a normal leaflet containing intercellular spaces. There was no histological evidence of changes in the midrib or major veins (fig. 3*B*), so that the increased prominence of these veins in gross aspect was probably a result of contrast with the darker green mesophyll. The darker color of the latter probably helped to obscure the minor veins, since they were not surrounded by an unpigmented parenchyma extending from the lower to the upper epidermis.

Laminae of leaflets from plants treated with 2,4-D developed internal structures by the activity of marginal meristems similar to the marginal meristems of controls (fig. 5*A*). These meristems laid down four internal layers of plate meristem (fig. 5*B*) similar to the corresponding layers in the controls. Thus, the six layers of plate meristem described for the embryonic leaves of controls were present in the embryonic leaves of treated plants.

As in the controls, maturation of tissues began at the tip of a leaflet. Figure 4*B* shows a cross section through the mature tissues at the tip of the same leaflet which had embryonic tissue at the level shown in figures 5*A* and 5*B*. The tissues of mature laminae of the earliest-formed leaflets of plants treated with 2,4-D resembled closely the comparable tissues from leaflets of plants treated with 2-chlorophenoxyacetic acid. In the later-formed leaves of plants treated with 2,4-D, fasciation of the veins was extensive. The vascular elements were discrete, but the unpigmented parenchyma surrounding the vascular elements of the midrib and major veins was continuous through much of the width of the leaflet. Chlorenchymatous tissue was confined to the margins and occasional islets surrounded by fasciated veins. There were no intercellular spaces in the chlorenchyma. Figure 5*C* shows a cross section from an extreme example of the smaller leaflets.

In the narrow, straplike leaflets of plants treated with 4-chlorophenoxyacetic acid, fasciation of the veins was

FIG. 4.—Section through (*A*) lamina of leaflet of plant treated with 2-chlorophenoxyacetic acid, illustrating failure to develop intercellular spaces; (*B*) tip of embryonic leaflet from plant treated with 2,4-D, illustrating maturity of tissue.

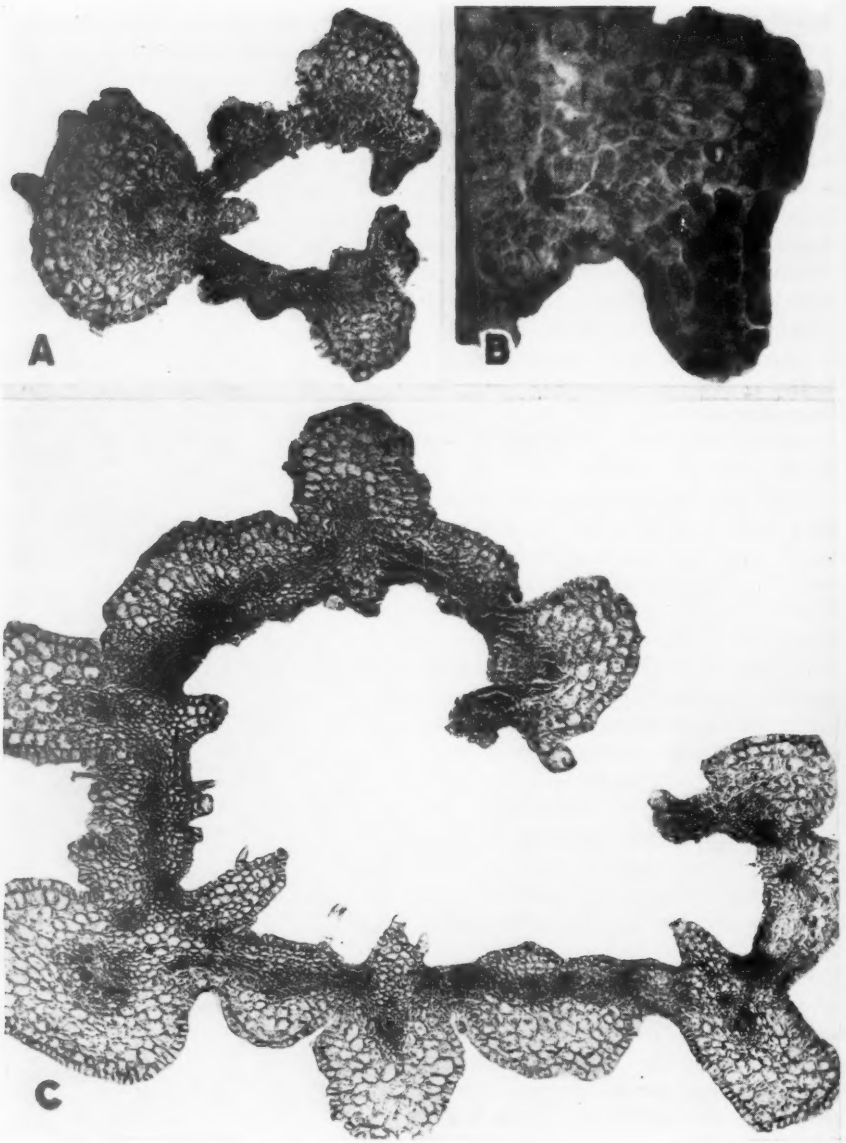

FIG. 5.—Cross sections of embryonic leaflet shown in fig. 4*B*, showing (*A*) origin of lamina and (*B*) details of marginal and plate meristems. *C*, cross section through extremely modified mature leaflet of plant treated with 2,4-D, showing fasciation of veins and chlorenchymatous tissue as dark-staining regions at margin and in islets between veins.

complete (fig. 6). Parenchyma surrounding the midrib and major veins was continuous from the upper to the lower epidermis. The parenchyma cells of the major veins contained no chlorophyll so that chlorenchymatous tissue was confined to a narrow margin of the leaflet. The vascular elements remained discrete and appeared as separate bundles imbedded in the parenchyma. Parenchyma cells farthest from the vascular elements were largest and those adjacent to the vascular elements were smallest. The vascular elements and the small parenchyma cells surrounding them formed a plate within the larger plate of parenchyma. At the extreme margin were a few chlorenchymatous cells that usually were confined to the layer corresponding to palisade in normal leaves.

Discussion

The gross responses observed in treated bean plants were similar to those previously reported. SWANSON (9) sprayed Red Kidney bean plants with 2,4-D and noticed epinasty and stem curvature in a few hours. The plants he observed also produced tiny leaves. BEAL (2, 3, 4) treated sweet pea, African mari-

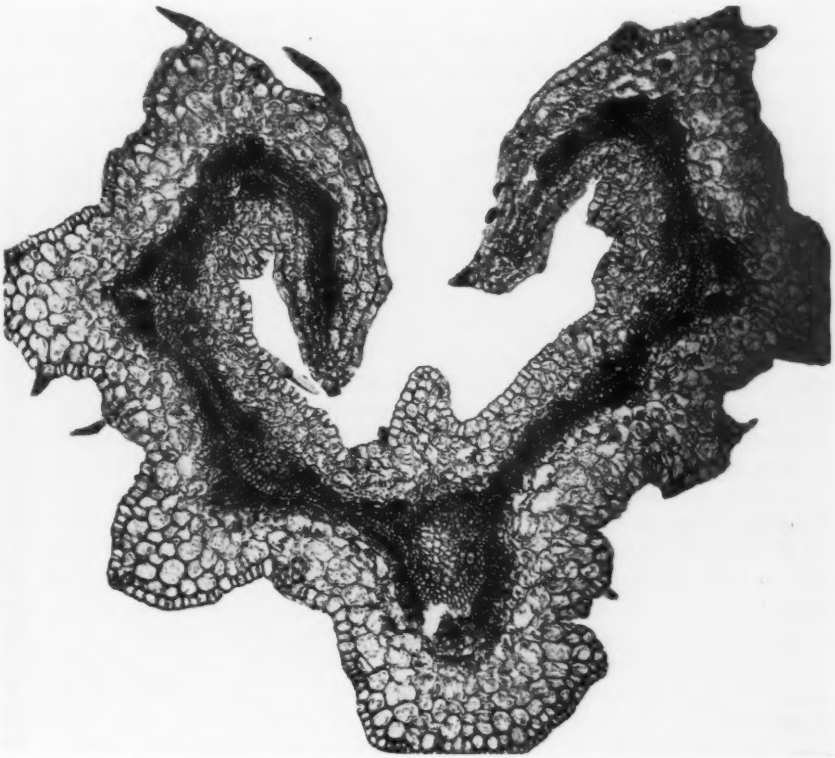

FIG. 6.—Cross section through narrow, rolled leaflet ultimately produced by plants treated with 4-chlorophenoxyacetic acid. Veins are fasciated, and vascular elements and small parenchyma cells surrounding them appear as darkly stained region across larger parenchyma region. Chlorenchyma confined to margin.

gold, and Red Kidney bean plants with substituted chlorophenoxy and other compounds. His experiments demonstrated that responses to treatment with 2-chlorophenoxyacetic acid applied in Carbowax were greater than when applied in lanolin. Conversely, 4-chlorophenoxyacetic acid applied in lanolin produced a marked response, although the same compound applied in Carbowax was ineffective. As in the present experiments, responses to 2,4-D were nearly indifferent to the carrier used.

MARTH and DAVIS (6) showed that temperature is of significance in the induction of responses to 2,4-D. If temperatures were too high, killing commonly resulted. In the present experiments concentrations of 0.5% of acid in the carrier were found to induce changes in the growth pattern at ordinary spring temperatures in a greenhouse. This concentration of 2,4-D and of 4-chlorophenoxyacetic acid was lethal at higher temperatures.

ZIMMERMAN and HITCHCOCK (12) reported formative effects of several phenoxy compounds on tobacco, tomato, and other plants. Some of their figures indicate progressive modification of leaves of plants treated with 4-chlorophenoxyacetic acid. The comparative stage of development of these leaves at the time of treatment is not clear. In the present study and in the experiments of BEAL (3), if the first trifoliate leaf was starting to emerge from the bud at the time of treatment with 4-chlorophenoxyacetic acid, its mature leaflets resembled the modified leaflets of plants treated with 2-chlorophenoxyacetic acid. Second and third trifoliate leaves, although usually similar in appearance, were much smaller. Subsequent leaves were almost uniform in size and had a narrow straplike form (fig. 1*E*). These later leaves were present only as primordia or perhaps were not yet differentiated at the time of treatment. This suggests that, if leaves are young enough, a uniform response to treatment with 4-chlorophenoxyacetic acid might occur. There was a suggestion of eventual uniformity of response in ZIMMERMAN and HITCHCOCK's report of successive whorls of fasciated leaves of tobacco treated with 4-chlorophenoxyacetic acid.

Modification in response to applications of 2,4-D produced a gradation in size that extended to leaves not yet apparent in the buds at the time of treatment. On the basis of the present experiments, it could not be determined whether the reduction in size of leaves developed following the application of 2-chlorophenoxyacetic acid was similarly progressive or whether the size gradation was a consequence of the relative immaturity of the later-formed leaves at the end of the experiments.

The development of the lamina of the untreated Red Kidney bean leaflet is in many respects similar to that observed in tobacco by AVERY (1). The subepidermal, marginal meristem in both plants gives rise to four layers within the epidermis. These layers remain four in the bean but become five in tobacco by division of one of the two inner layers. In both plants the adaxial of these four internal layers become palisade parenchyma, while the other three internal layers (four in tobacco) differentiate into spongy mesophyll. Subsequent to the formation of the four layers of plate meristems in bean leaflets and the five layers in tobacco leaves, all cell divisions produced new cells in the plane of the plate meristems, except those involving vein formation. The divisions in this

plane led SCHUEPP (7) to term these layers "Plattenmeristem." SMITH (8) showed that the constancy in the number of layers, and hence extension of the lamina by growth of plate meristem, is of regular occurrence in embryonic leaves with the exception of certain sun leaves.

Veins of the minor vascular network in bean leaflets originate in the second layer beneath the adaxial epidermis. AVERY (1) stated that this is the usual mode of origin in tobacco. FOSTER (5), in his summary, showed diagrams indicating that veins usually originate in the layer immediately beneath the embryonic palisade or palisades.

According to AVERY (1), the development of intercellular spaces in mesophyll is a result of continued cell enlargement in the epidermis after cell division and enlargement in the mesophyll have ceased. The compounds used in the present experiments inhibited the formation of intercellular spaces. This possibly indicated that the epidermal cells of the laminae of bean plants treated with substituted phenoxy compounds failed to enlarge. There is also the possibility that lysis of the middle lamellae of mesophyll cells failed to occur. This would render epidermal cell enlargement ineffective in producing intercellular spaces. Since the mesophyll cells then would not separate, the epidermal cells would be forced to increase their volume by periclinal expansion.

The narrow leaflets of plants treated with 4-chlorophenoxyacetic acid indicated supression of the activity of the plate meristem. The resulting approximation of veins resulted in fasciation. The parenchyma surrounding the veins became continuous although the vascular elements remained discrete. The laminae of leaflets from plants treated with 2,4-D exhibited an internal structure in the earlier leaves similar to that induced by 2-chlorophenoxyacetic acid. The gradation from this form to leaflets resembling those from plants treated with 4-chlorophenoxyacetic acid has been noted. This indicated a progressive inhibition of cell division in the plate meristem, for the laminal meristem was present and functional.

Summary

1. A bean leaflet develops a lamina by the activity of a subepidermal marginal meristem, which produces four internal layers of plate meristem. The adaxial of these layers develops into the palisade layer; the other three produce the spongy mesophyll.

2. Veins of the minor network are initiated by divisions of a row of cells in the layer beneath the embryonic palisade. Additional cells and additional layers become involved.

3. Treatment of young plants with 0.5% concentration of 2-chlorophenoxyacetic acid in Carbowax inhibited the formation of intercellular spaces in the mesophyll of bean leaflets.

4. Similar application of 0.5% 4-chlorophenoxyacetic acid in lanolin inhibited the activity of plate meristem in the laminae of bean leaflets. As a result, veins were approximate and their parenchyma became continuous although the vascular elements remained discrete. Chlorenchymatous tissue was confined to the margin.

5. Similar treatment with 0.5% concentrations of 2,4-dichlorophenoxyacetic acid, in either lanolin or Carbowax as the carrier, induced progressive modification of bean leaves. The earliest leaves were similar to those induced by treatment with 2-chlorophenoxyacetic acid. In later

leaves the external form and internal structure resembled that found in leaflets of plants treated with 4-chlorophenoxyacetic acid.

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COMPARATIVE HERBICIDAL VALUE OF 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4,5-TRICHLOROPHENOXYACETIC ACID ON SOME HERBACEOUS WEEDS, SHRUBS, AND TREES UNDER HAWAIIAN CONDITIONS[1]

R. K. TAM

Introduction

The use of 2,4-dichlorophenoxyacetic acid (2,4-D) as a herbicide has been emphasized in nearly all investigations since the early reports of MARTH and MITCHELL (**7**) and HAMNER and TUKEY (**3**), although the possible use of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) for similar purposes was recognized by HAMNER and TUKEY (**2**). With the kidney bean and soybean plants, SWANSON (**8**) showed that 2,4,5-T and most of its derivatives, carried in oil, produced a greater inhibitory effect, in general, than 2,4-D. Another instance of the greater toxicity of 2,4,5-T and of other compounds with the 2,4,5-trichlorophenoxy-configuration was observed by ENNIS *et*

[1] Published with the approval of the Director as Miscellaneous paper no. 45 of the Pineapple Research Institute.

al. (1) when tested on the Irish potato in both water and oil solutions.

In the last 2 years both 2,4-D and 2,4,5-T have been sprayed on many herbaceous weeds, shrubs, and trees growing under warm humid Hawaiian conditions. The investigations of VAN OVERBEEK and VELEZ (9) and of VAN OVERBEEK *et al.* (10) with 2,4-D under somewhat similar conditions are recognized. It is the purpose of this report to indicate the responses of some of these plants to 2,4-D and to point out several species of plants in which better kills resulted with 2,4,5-T.

Material and methods

Preliminary tests confirmed the observations of HAMNER and TUKEY (4) that, although solutions of 500 and 1000 p.p.m. of the compounds were acceptable for herbicidal use on some species of herbaceous weeds, higher concentrations (2000–5000 p.p.m.) were desirable for woody plants. For all the tests reported here, the acid forms of 2,4-D and 2,4,5-T were used, while conversion to the sodium and ammonium forms was often necessary to increase their solubility in water.

When aqueous solutions of 1000 p.p.m. or less were made up, Carbowax 1500 was used as the carrier. Solutions of 2000–10,000 p.p.m. were prepared by forming the sodium salt, using an equal amount of sodium bicarbonate; ammonium carbonate was used to form the ammonium salt. Wetting was enhanced by adding water-soluble Standard Wetting Agent (a sodium sulfonated aromatic prepared by the Standard Oil Company of California) at the rate of 2 lb./100 gal. dilute spray.

Diesel oil (Pacific Standard 200) solutions of 1000–20,000 p.p.m. (2.0%) of both 2,4-D and 2,4,5-T were prepared by dissolving the acids in diethylene dioxide (1,4 Dioxane-practical) and by adding diesel oil up to 80% of the final solution. 2,4-D for testing was supplied by the American Chemical Paint Company, the Dow Chemical Company, and E. J. KRAUS. 2,4,5-T was obtained from J. W. MITCHELL, the American Chemical Paint Company, and the Dow Chemical Company. Maximum solubilities of samples of the same chemical obtained from the different sources have proved not to be equal under all conditions. Each sample was used only when complete solubility provided the desired concentration. Whenever possible, solutions were made up and used the same day.

Under Hawaiian conditions, a daily range in temperature from a minimum of 70° F. to a maximum of 85° F. occurs during a large percentage of the days in the year. There are few days in which temperatures range from 60° to 70° F., and this range is usually confined to locations of higher altitude. Prolonged periods of this range are rare. Suitable temperatures for the activity of the chemicals used are usually encountered the year round, being above the 50°–60° F. range in which retardation of kill has been shown to occur (6).

Rainfall under these subtropical conditions is a greater problem in the use of the herbicides. Heavy dew and some rainfall (especially in the upland areas) prevail for many nights and days from October to April. It was noted in several early tests that response to treatment was limited when light showers fell 6–24 hours after application. In one instance total lack of response occurred when a moderate rain fell within 2 hours after applying the chemicals. A prolonged period of high humidity for several weeks after treatment with 2,4-D and 2,4,5-T has resulted, in one case, in heavy forma-

tion of aerial roots on stems of several species of mature weeds. Despite severe twisting and swelling of stems, complete kill under these conditions was not obtained. Spraying of 2,4-D or 2,4,5-T in oil under these moist conditions, as suggested by WEAVER *et al.* (11), may render their action more effective.

Results

HERBACEOUS AND SEMIWOODY WEEDS.—Weeds growing under natural conditions in the field were sprayed with 1000 p.p.m. of 2,4-D and the same concentration of 2,4,5-T. Volume of spray applied was 160 gal./acre. Weather following spraying was dry for at least 24 hours, and during the period of study the mean daily temperature averaged above 70° F. Total damage, as estimated from desiccation and defoliation of several plants, was reported as mean percentage kill (table 1).

Groups A and B in table 1 include a large percentage of the total population of common herbaceous and semiwoody

TABLE 1

PERCENTAGE KILL OF HERBACEOUS AND SEMIWOODY WEEDS, FOUND IN HAWAIIAN PINEAPPLE FIELDS, 6 WEEKS AFTER SPRAYING WITH AQUEOUS SOLUTIONS (1000 P.P.M.) OF 2,4-D AND 2,4,5-T

Species	2, 4–D	2, 4, 5–T
A. *Species equally affected by 2,4-D and 2,4,5-T:*		
Balsam apple (Momordica balsamina L.)	100	100
Field bindweed (Convolvulus arvensis L.)	100*	100*
Kikania, cocklebur (Xanthium pennsylvanicum Wallr.)	100	100
Castor bean (Ricinus communis L.)	100	100
Hialoa (Waltheria americana L.)	100	100
Wild indigo (Indigofera suffruticosa Mill.)†	100	100
Nettle-leaved goosefoot (Chenopodium murale L.)	100	100
Asiatic pennywort (Centella asiatica [L.] Urban)	100	100
Spanish needles (Bidens pilosa L.)	100	100
Horseweed (Erigeron albidus [Willd.] Gray)	100	100
Purslane (Portulaca oleracea L.)	100	100
Richardsonia (Richardsonia scabra [L.] St. Hil.)	100	100
B. *Species more affected by 2,4-D than by 2,4,5-T:*		
Swine's-cress (Senebiera didyma Persoon)	100	75
Cheese weed (Malva rotundifolia L.)	95	25
False mallow (Malvastrum coromandelianum [L.] Garcke)	100	25
Ilima (Sida fallax Walp.)	100	50
False ragweed (Franseria strigulosa Rydb.)	95	50
Lamb's-quarters (Chenopodium album L.)	100	75
Red pualele, Flora's-paintbrush (Emilia sonchifolia DC)	100	75
Nutgrass (Cyperus rotundus L.)	100*	95*
Honohono (Commelina nudiflora L.)	100	95
C. *Species more affected by 2,4,5-T than by 2,4-D:*		
Popolo (Solanum nodiflorum Jacq.)	35	100
Hilahila, sensitive plant (Mimosa pudica L.)	50*	75*
Wood sorrel (Oxalis martiana Zucc.)	50*	100*
Phyllanthus weed (Phyllanthus niruri L.)	25	95
Christmas berry (Schinus terebinthifolius Raddi)	50	100
D. *Species not killed by either 2,4-D or 2,4,5-T:*		
Jimson weed (Datura stramonium L.)	25	25
Ageratum (Ageratum conyzoides L.)	50	50

* May require two or more treatments to effect control, owing to formation of new shoots.
† Young plants (3–6 in. tall).

broad-leaved weeds found in pineapple fields and wayside places. For the control of species listed in these two groups, it does not seem advantageous to use 2,4,5-T, since 2,4-D proved either equally or more toxic (fig. 1). Following treatment of areas heavily infested with nutgrass (*Cyperus rotundus*), new shoots appeared more rapidly and in greater number with 2,4,5-T than 2,4-D. In special tests it was found that repeated sprayings, preferably with a 5000-p.p.m. aqueous solution, were necessary for reducing the population of this weed in in-

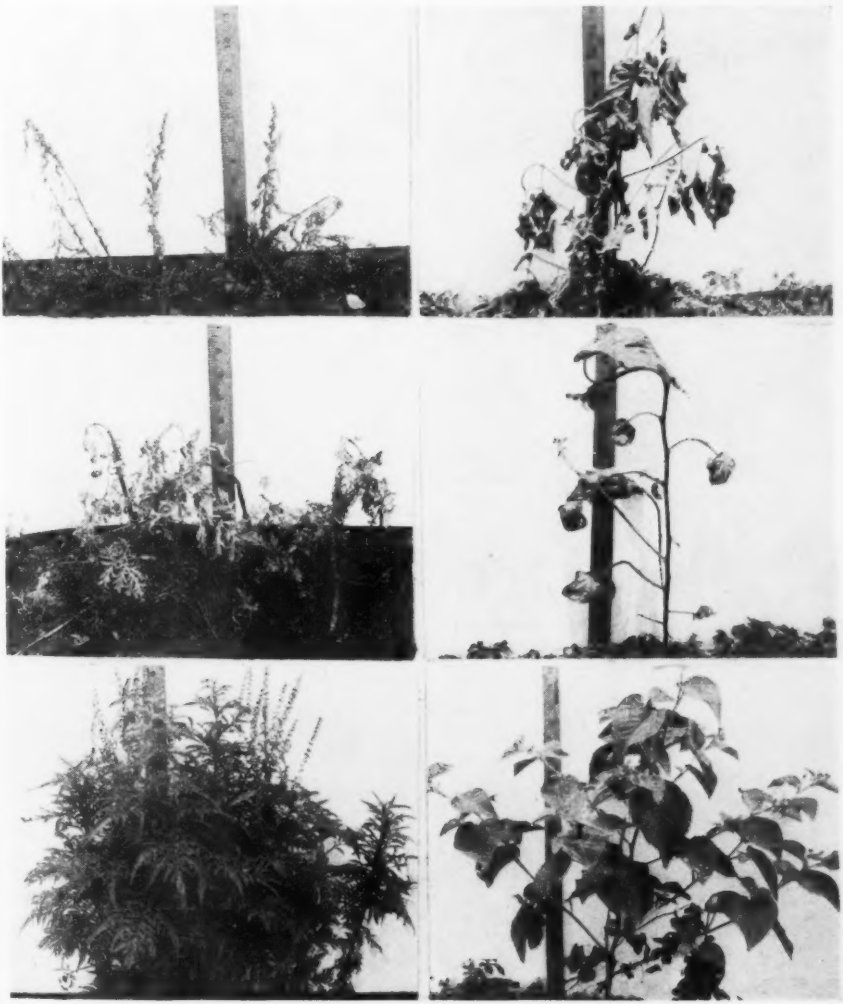

FIG. 1.—Greater toxicity of 2,4-D (*upper*) than of 2,4,5-T (*center*); applied as aqueous sprays (1000 p.p.m.) 6 weeks earlier. *Lower*, untreated. *Left, Franseria strigulosa; right, Sida fallax.*

fested areas. Oil solutions of 2,4-D rapidly scorched plants, but new stands were quickly formed.

Group C contains five species of weeds which appeared to be more affected by 2,4,5-T than 2,4-D when plants were sprayed with these chemicals at a concentration of 1000 p.p.m. Of these, popolo (fig. 2) and Christmas berry were shown, in several subsequent tests using higher concentrations of the chemicals, to be killed by 2,4,5-T while being unusually resistant to the activity of 2,4-D. In this first test, the Christmas berry

FIG. 2.—Greater toxicity of 2,4,5-T (*center*) than of 2,4-D (*upper*); applied as aqueous sprays (1000 p.p.m.) 6 weeks earlier. *Lower*, untreated. *Left, Eucalyptus citriodora; right, Solanum nodiflorum.*

plants were seedlings about 6 inches tall; the high toxicity of 2,4,5-T to this species was also indicated in other tests with mature trees 10–15 feet tall in which one spraying often resulted in complete kill. Plants in group D were not killed by either 2,4-D or 2,4,5-T.

SHRUBS AND TREES.—There are several species of shrubs and trees which infest valuable pasture land, and the extermination of some of these is highly desirable. Among the most serious and widespread are guava and lantana. 2,4,5-T was more toxic than 2,4-D to all species of woody shrubs and trees tested, with the exception of guava (table 2). Lantana was not greatly affected by 1000-p.p.m. solutions of 2,4-D and 2,4,5-T, although 2,4,5-T rated higher than 2,4-D. This was confirmed in later tests using stronger concentrations. The resistance of popolo to 2,4-D, as reported in table 1, was also noted in this test. *Eucalyptus citriodora* (fig. 2), when young was killed readily by 1000 p.p.m. of 2,4,5-T, while the same concentration of 2,4-D proved comparatively innocuous. The striking selectivity was further demonstrated in mature trees, 40–50 feet tall, which had been completely girdled 3 feet above the ground and painted on the wounds with 15% solutions of the chemicals in Carbowax 1500. When 2,4,5-T was applied, complete kill resulted, with 100% desiccation of foliage in about 6 months. In 2,4-D treated trees and in girdled but untreated trees excessive gum exudation followed with no visible systemic injury to the trees even after 20 months. In the case of false koa, new growth following complete defoliation was rapid; this is fortunate, since this plant is considered valuable as forage. The algaroba or kiawe (fig. 3) responded to both compounds, with 2,4,5-T slightly more toxic.

A later test was installed to check the toxicity of 2,4,5-T, including other woody pests not previously tested. An area of about 1 acre was sprayed with an aqueous solution of 2000 p.p.m. of 2,4,5-T. The high toxicity of this compound to the shrubs and trees listed in table 3 was undoubtedly influenced partially by continued warm and dry weather during this test. With the exception of pluchea, complete desiccation of nearly all the plants in the test area was evidenced. Subsequent formation of new shoots in some isolated instances, in which coverage by spraying was possibly incomplete, would have necessitated only a limited amount of spot spraying for complete control.

TABLE 2

PERCENTAGE TOXICITY TO SOME SHRUBS AND TREES OF AQUEOUS SOLUTIONS (1000 P.P.M.) OF 2,4-D AND 2,4,5-T, 6 WEEKS AFTER SPRAYING

Species	2,4-D	2,4,5-T
Guava (Psidium guajava L.)	95*	90*
Lantana (Lantana camara L.)	25*	50*
False ironwood (Casuarina equisetifolia L.)	90*	100*
Eucalyptus (Eucalyptus citriodora Hooker)	25*	100
Algaroba, kiawe (Prosopis chilensis [Molina] Stuntz)	95	100
False koa (Leucaena glauca [L.] Benth.)	75*	100*
Popolo (Solanum nodiflorum)	25	100

* Requires two or more sprayings, since new shoots appeared 2–3 months after first application.

Recovery was noted to be more consistent and more rapid in lantana. This species may require even more concentrated solutions of 2,4,5-T for complete control with a single application. Defoliation following treatment was 100% in this species; it was noted, in all tests conducted to date, that regeneration is most likely in those species in which defoliation takes place in response to treat-

ment. When 100% desiccation of leaves occurs with no noticeable defoliation for a long period following spraying, a complete kill of the large woody plants tested is practically a certainty. It was also noted that, in cases in which special care had been taken to wet all the stem surfaces above ground, chances of a complete kill were greater than if only the leaves were sprayed.

Results from the foregoing tests showed that, despite desiccation and complete defoliation with some species, notably lantana and guava, complete kill was often not obtained with one spraying of the dilute chemicals. It was thought that a complete kill might be accomplished by a high concentration of the chemicals carried in oil. Subsequently, several species, including mostly large mature plants, were sprayed with 2.0% solutions of 2,4-D and 2,4,5-T (table 4). The selectivity of 2,4-D for guava and of 2,4,5-T for eucalyptus was not evident when both chemicals were used in the high concentration of 2.0% in diesel oil. Against lantana, which has proved to be one of the hardest plants to kill, 2,4,5-T again proved to be more toxic than 2,4-D, even in high concen-

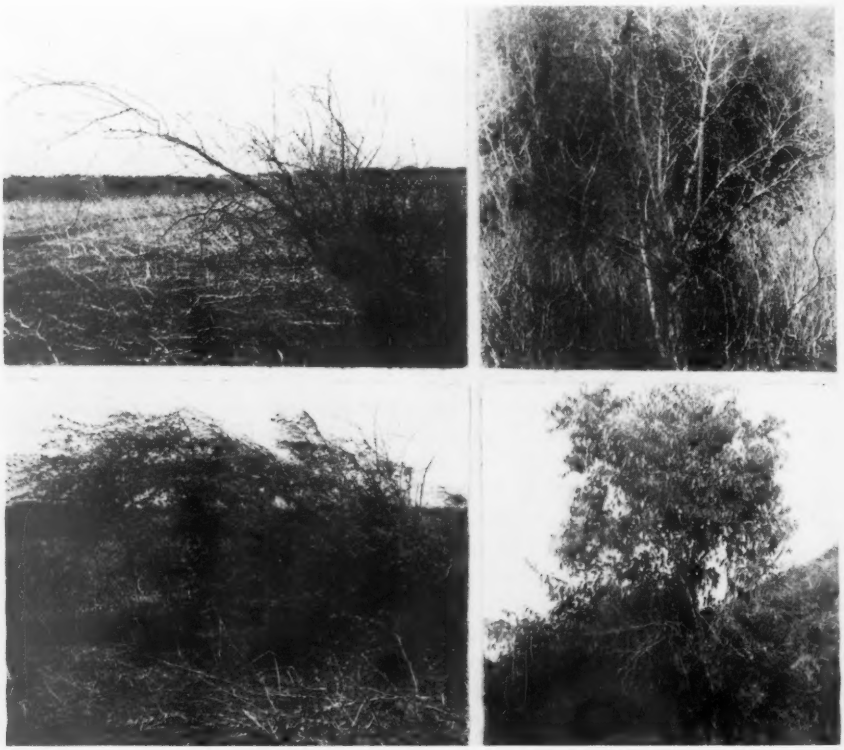

FIG. 3.—Toxicity of 2,4,5-T to (*upper left*) *Prosopis chilensis* and (*upper right*) *Eugenia jambolana*, applied as aqueous spray (2000 p.p.m.) 12 and 6 weeks earlier, respectively. *Lower left*, same *Prosopis* before spraying; *lower right*, untreated *Eugenia*.

trations. Use of these chemicals in high percentage and low volumes may find practical application on these and other resistant plants.

PINEAPPLES.—The possibility of using such herbicides for weed control in fields of growing pineapples was investigated (table 5). When sprayed at the rate of 160 gal./acre, concentrations of 2,4-D up to 10,000 p.p.m. did not completely kill 8-month-old pineapple plants. With both sodium and ammonium salts of 2,4-D, a maximum toxicity of 95% was indicated at the highest concentration. Formation of new ground suckers proved that, with some plants, portions of the stem below the soil surface were not killed. In contrast to the low toxicity of both sodium and ammonium 2,4-D at 2000 p.p.m., 2,4,5-T in the same concentration was at least 95% toxic. Use of solutions of 5000 and 10,000 p.p.m. of 2,4,5-T resulted in 100% kill. The inability of the plants treated with 2,4,5-T to form new ground suckers indicated 40% toxicity, as in the case of 2000 p.p.m. of 2,4-D, precludes the use of this type of weed-killer as a spray in pineapple fields.

Discussion

In tests which compared the effects of 2,4-D and 2,4,5-T on plants growing in a

TABLE 3

PERCENTAGE DESICCATION AND DEFOLIATION OF SHRUBS AND TREES 6 WEEKS AFTER SPRAYING WITH 2,4,5-T (2000 P.P.M.)

Species	Desiccation	Defoliation
Christmas berry (Schinus terebinthifolius)	100*	0
Java plum (Eugenia jambolana Lamarck)	100*	50
Lantana (Lantana camara)	100*	100
Pluchea (Pluchea odorata [L.] Cass.)	50*	50
Castor bean (Ricinus communis)	100	75
Cat's-claw (Caesalpinia sepiaria Roxb.)	100*	100
Eucalyptus (Eucalyptus citriodora)	100	0
False ironwood (Casuarina equisetifolia)	100*	0

* May require two or more sprayings, since in some cases with large plants new shoots appeared 2 or 3 months after first application.

TABLE 4

PERCENTAGE KILL OF SPECIES OF SHRUBS AND TREES 12 WEEKS AFTER APPLICATION OF 2.0% 2,4-D AND 2,4,5-T IN 20% DIOXANE AND 80% DIESEL OIL

Species	2,4-D	2,4,5-T	Diesel oil control
Guava (Psidium guajava)	100	100	25*
Lantana (Lantana camara)	95*	100	25*
Eucalyptus (Eucalyptus citriodora)	100	100	10*
False ironwood (Casuarina equisetifolia)	100	100	75*
Wild indigo (Indigofera suffructicosa)†	100	100	.....

* New shoots appeared. † Mature plants 3–4 ft. tall.

that not only were the parts of the plant above the ground destroyed but also that the portions of stem below the ground were rendered incapable of regeneration. On the basis of these results, 2,4,5-T in low concentration appeared to be approximately twice as toxic to pineapples as 2,4-D. However, even a semitropical region, results indicated that with some species better kills were obtained with 2,4,5-T than with 2,4-D. These were, in general, plants classified as shrubs and trees. With the more susceptible, small, common herbaceous weeds of lawn and field, 2,4-D was either more effective, or, as in most cases, little

difference was found between it and 2,4,5-T.

Eucalyptus citriodora is an outstanding example of a species to which 2,4,5-T is more toxic than 2,4-D. When it was sprayed with aqueous solutions at 1000–2000 p.p.m., it was little damaged by 2,4-D, whereas 2,4,5-T gave complete kills with only one spraying on plants 5–10 feet tall. This differential toxicity was further demonstrated when trees 40–50 feet tall were girdled and concentrated Carbowax solutions of the two compounds were applied to the cut area. 2,4,5-T treatment resulted in complete kill in 6 months, while plants girdled and untreated or treated with 2,4-D were alive after 20 months. Only when plants were completely covered with 2.0% 2,4-D and 2,4,5-T sprays in diesel oil was the selectivity of 2,4,5-T for eucalyptus not in evidence; under these conditions both 2,4-D and 2,4,5-T produced 100% kills.

Although the herbicidal value of 2,4-D for most herbaceous weeds has been extensively investigated and confirmed, it seems that future trials with 2,4,5-T, especially on the woody types which are hard to kill, would furnish valuable information on its possible use as a supplement to 2,4-D in a general weed-control program. The use of 2,4,5-T in oil seems advantageous, since high concentration is possible, and, coupled with high or low pressures and special nozzles (**5**), atomization makes it possible to spray large trees with small volumes per acre. The greater inhibitory effect per unit of 2,4,5-T or its derivatives (**8**) in oil, plus the possible minimizing of the deleterious effects of rainfall by this method (**11**), should prove valuable under most conditions and especially under the moist subtropical Hawaiian conditions.

TABLE 5

TOXICITY TO 8-MONTH-OLD PINEAPPLE PLANTS OF SODIUM 2,4-D, AMMONIUM 2,4-D, AND AMMONIUM 2,4,5-T IN AQUEOUS SOLUTION, SPRAYED AT RATE OF 160 GAL./ACRE. READINGS 3 MONTHS AFTER APPLICATION

Treatment (no.)	Chemical	Concentration (p.p.m.)	Toxicity (%)	Ground suckers (no.)
1	Sodium 2,4-D	2,000	40	.....
2	Sodium 2,4-D	5,000	75	.....
3	Sodium 2,4-D	10,000	95	2
4	Ammonium 2,4-D	2,000	55	.....
5	Ammonium 2,4-D	5,000	75	.....
6	Ammonium 2,4-D	10,000	95	5
7	Ammonium 2,4,5-T	2,000	95	0
8	Ammonium 2,4,5-T	5,000	100	0
9	Ammonium 2,4,5-T	10,000	100	0

* Mean of forty plants in each treatment.

Summary

1. Some species of herbaceous and semiwoody weeds, shrubs, and trees were sprayed with 1000–2000 p.p.m. aqueous solutions of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) under warm humid Hawaiian conditions. In addition, several woody species were sprayed with

2.0% concentrations of the chemicals in diesel oil.

2. When sprayed on species of herbaceous or semiwoody weeds, 2,4-D was either greater than, or equal to, 2,4,5-T in toxicity in aqueous solution at 1000 p.p.m.

3. When tested on some species of shrubs and trees, 2,4,5-T was observed to be more toxic than 2,4-D. Woody species, such as eucalyptus (*Eucalyptus citriodora*), popolo (*Solanum nodiflorum*), sensitive plant (*Mimosa pudica*), Christmas berry (*Schinus terebinthifolius*), lantana (*Lantana camara*), false ironwood (*Casuarina equisetifolia*), algaroba (*Prosopis chilensis*), Java plum (*Eugenia jambolana*), and cat's-claw (*Caesalpinia sepiaria*) were more injured by 2,4,5-T treatment. The guava (*Psidium guajava*) was an exception, being damaged more by 2,4-D than by 2,4,5-T.

4. Eucalyptus was killed with one spraying of 1000 p.p.m. of 2,4,5-T, while the same concentration of 2,4-D did little damage. The specificity of 2,4,5-T for this species was further demonstrated with mature trees, 40–50 feet tall, when girdled trees were killed in 6 months after 2,4,5-T was applied to the wounds, while plants treated with 2,4-D were alive 20 months after treatment.

5. With the exception of lantana, which proved to be one of the most difficult species to control, 2.0% solutions of 2,4-D and 2,4,5-T in diesel oil proved to be equal in toxicity to several woody species tested. Although the specificity of 2,4,5-T for eucalyptus was not in evidence at this high concentration, lantana was destroyed with one spraying of 2,4,5-T while 2,4-D–treated plants required repeated sprayings for control.

6. When sprayed on 8-month-old, actively growing pineapple plants, ammonium 2,4,5-T in aqueous solution at 2000–10,000 p.p.m. proved practically 100% toxic, while similar solutions of 2,4-D possessed much lower toxicity. In these tests 2,4,5-T was approximately twice as toxic as 2,4-D, but the damage produced by both compounds precludes their use as herbicidal sprays in fields of growing pineapples.

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STUDIES ON THE CAROTENE-DESTROYING PROCESSES IN DRYING BEAN LEAVES

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 595

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Introduction

It is generally recognized that field-curing of hay crops results in large losses of carotenoid pigments (1, 8, 12). These losses have been variously ascribed to the action of an enzyme and to light, different investigators emphasizing one or the other mechanism. HAUGE and co-workers (5, 6, 9, 10) have maintained that enzymic activity is mainly responsible for carotene loss, while GUILBERT (4) has emphasized the importance of light.

The terms "enzymic destruction" and "photodestruction" of carotene will be used in this paper. It is realized that if the enzyme involved in this process is a lipoxidase, as MITCHELL and HAUGE believe (9), then there is a coupled oxidation involving fats (14, 16) and not a direct enzymic oxidation with carotene as the substrate. It will be shown that photodestruction is likewise indirect, involving at least the absorption of light by substances other than the carotenoids. For brevity, however, the indirect nature of the processes will not be referred to again in naming them.

MITCHELL and HAUGE (10) measured the carotene loss in alfalfa drying in the light and in the dark in order to determine the relative importance of enzymic destruction and light-activated destruction. Although they had to assume that enzymic destruction of carotene was the same in the light and in the dark, they realized the weakness in their assumption and enumerated factors which would cause differences in enzymic destruction between light and dark. It is impossible, therefore, to reach any conclusion as to the amount of carotene destroyed by light.

GUILBERT (4) compared the loss of carotene in sun-curing of autoclaved and unautoclaved alfalfa and concluded that photodestruction was responsible for a large fraction of the loss. MITCHELL and HAUGE claimed (10), however, that autoclaving affects the stability of carotenoids to subsequent exposure to light, so that it is not valid to compare carotene losses in autoclaved and unautoclaved leaves in the light as a means of determining the photo-oxidative loss. A further objection is that photodestruction is effective in autoclaved leaves from the moment of exposure to light, since the cells are already killed, whereas in fresh leaves a period must elapse during which the cells are killed or injured by partial drying before photodestruction (and enzymic destruction) becomes operative (see below).

With the restricted validity of the above-mentioned experimental techniques in mind, the present work was undertaken. Duplication of field conditions was sacrificed in favor of controlled environments, so that for specified conditions, at least, the extent of photodestruction and enzymic destruction could be quantitatively determined. The effects of varying environmental and internal factors—including the time factor, temperature, oxygen tension, light in-

tensity, light quality, water content of the leaf, and the presence or absence of naturally occurring, water-soluble antioxidants—on the rates of photodestruction and/or enzymic destruction have been investigated.

Material and methods

The leaves of Red Kidney bean plants were chosen as the experimental material because of their ready availability and because of the ease in preparing uniform samples, especially when orientation of the leaves to light is an important factor. Cordate leaves of plants grown in greenhouse soil to the stage when these leaves are fully expanded were used. The half-leaf method was employed. By cutting out the midrib, two sets of matched half-leaves were obtained; one was used as a control, the other as a treatment. Ten leaves sufficed to give matched sets which agreed within the limit of the chromatographic technique employed for carotenoid determinations. Analysis of six pairs of sets (ten half-leaves per set) gave a mean difference between matched sets of 1.0 ± 0.3 microgram of carotene per gram of fresh weight (less than 1% of the carotene content), with a maximum difference of 2.1 μg/gm. (1.7%).

The chromatographic method employed was essentially that of ZSCHEILE and WHITMORE (**19**) for fresh plant material. Modifications of the method included complete transfer of pigments from the aqueous phase to Skellysolve following extraction and the use of calcium hydroxide (approximately 1–2 cm. thick) to overlay the magnesia in the column. The calcium hydroxide gave better retention of the chlorophylls and allowed for elution and measurement of carotenols by 40% acetone in Skellysolve, following the elution of carotenes, without contamination by chlorophylls.

The term "carotenol" as used in this paper is equivalent to the more generally used term "xanthophyll." The carotenol fraction consisted of approximately 80% luteol, the remainder including two or more other carotenol pigments. Carotenol values were calculated from the standard carotene curve and are of significance for comparative purposes only.

The criterion for carotene destruction was decolorization of carotene as measured at 440 mμ according to the method of ZSCHEILE and WHITMORE.

Treatment of leaves by freezing, autoclaving, or drying prior to exposure to experimental conditions was necessary to insure controlled initiation of carotene destruction at the start of the experiment.

Our observations indicate that at least intensive injury, if not death, of cells is necessary for any marked carotene destruction. Conditions such as freezing or autoclaving which kill leaves, or a degree of dehydration sufficient to cause injury, are necessary pretreatments for rapid enzymic destruction and/or photodestruction of carotene. It has been postulated that the change in cells upon death which accounts for the increased susceptibility of carotene to enzymic destruction is due to increased contact between enzyme and lipoidal constituents resulting from disorganization of cell structure (**13**, p. 117).

Of the treatments employed, predrying has the advantage of being more comparable with field-curing conditions than either freezing or autoclaving. Prefreezing has an advantage over predrying, however, because there is no loss of carotene in pretreatment and cells are uniformly killed, resulting in more or less equal enzymic activity throughout the leaf. Preautoclaving has an advantage over predrying in that the cells are

uniformly killed and the enzymes inactivated.

Leaves were predried at room temperature (22° C.) in a vacuum oven containing a generous supply of anhydrous calcium chloride, so that complete dryness could be obtained in 1½–2 hours. In this manner, carotene loss before the leaves had reached a given moisture content was kept small (5% of original carotene content), and destruction of carotene could be initiated at a precise time. Prefreezing was accomplished by cooling the leaves to −18° C. for 16 hours. Autoclaved leaves were heated at 5 pounds pressure in an autoclave (108° C.) for 5 minutes.

In the studies on photodestruction the leaves were irradiated by a mercury vapor lamp (400-watt, type H-1, G.E.) equipped with a water filter. The incident light intensity usually employed was between 1000 and 1500 foot-candles. In the studies on light quality a 1000-watt tungsten-filament lamp was substituted as the light source. The leaves either were floated on water to prevent drying during exposure or more usually were dipped in heavy, white mineral oil and spread out in an open dish. The oil had no effect on rates of enzymic destruction or photodestruction as determined by comparison with leaves in moist-chambers. Exposure of leaves not covered by mineral oil in moist-chambers resulted in large losses of water from the leaves owing to slight heating of the irradiated leaves above air temperature. Leaves dipped in mineral oil in open dishes lost water equivalent to less than 10% of their fresh weight during 6 hours of exposure to 1300 foot-candles. At the end of the experiment most of the oil was removed by rinsing the leaves lightly in Skellysolve B. Control sets (kept in the dark during the experimental period) received the same mineral-oil treatment.

In experiments with predried or prefrozen leaves, dark controls were used to correct for the enzymic destruction which occurred in sets exposed to light. With autoclaved material, control sets in the dark served to correct for a nonenzymic oxidation of carotene which, under the conditions of time and temperature employed, was in most cases negligible. Loss in carotenoid pigment is expressed either on an absolute basis or as a percentage of initial carotene content (as determined in control sets of autoclaved samples, or in separate samples, where controls were used to determine enzymic destruction).

All samples which were not autoclaved prior to experimental exposure were blanched in boiling water for 2 minutes at the conclusion of the run. All samples were stored at −18° C. until analyzed.

Experimentation

PRELIMINARY EXPERIMENT.—In one of our first attempts to evaluate the relative importance of enzymic destruction and photodestruction of carotenoids, fresh and autoclaved leaves were exposed to sunlight (6000 foot-candles), while comparable samples were kept near by in the dark (same air temperature). The exposed sets (light and dark) were taken up at intervals and re-weighed to determine moisture loss. Carotene loss in exposed sets was calculated as a percentage of initial carotene content.

In table 1 data are grouped together according to the amount of water lost, and the mean carotene loss for each group is reported. Fresh leaves lost approximately 30% of their carotene in the dark, while autoclaved leaves lost only about 1%, indicating that an enzyme-mediated destruction of carotene exists. This conclusion is based on the assumption that the only effect of autoclaving

pertaining to carotene destruction is in inactivating enzymes, although other explanations have been advanced (**13**, p. 116; **18**). Autoclaved leaves in the light lost 53% of their carotene in 2–3 hours, while comparable leaves in the dark lost only about 1% up to 29 hours. This proves that a light-activated destruction of carotene can occur.

In fresh leaves in the light, both the enzyme and the light-activated mechanisms are responsible for the destruction of carotene during drying, but it is impossible from these data to calculate what part of total destruction was due to each mechanism. Fresh leaves in the dark cannot be used in measuring the extent of enzymic destruction which occurred in the light, because differences in leaf temperature and moisture content at any given time could have caused the amount of enzymic destruction in the light to differ from that in the dark. For reasons already mentioned, autoclaved leaves in the light cannot serve in measuring photodestruction in fresh leaves in the light. In an attempt to obviate these difficulties, simulated field conditions (gradual drying of material) were sacrificed in favor of precisely controlled internal and external factors.

TIME-RATE STUDIES.—Knowledge of the influence of time on the rate of destruction of carotene is essential for the study of other factors affecting the process. A preliminary experiment showed that maximum enzymic destruction of carotene occurred when leaves had lost 50–90% of their water content. In another experiment leaves were dried at room temperature until 85–91% of the moisture had been evaporated and then incubated at 37° C. for various periods of time, allowing no further change in moisture content. Control samples were blanched after the dehydration process. Curve *B* (fig. 1) presents these data, which show a large initial rate of carotene loss and subsequent decrease in rate. This results in leveling-off of the carotene loss curve in the region in which 40–50% of the carotene has been destroyed. The change in rate of carotene loss necessitates using an incubation period of 2 hours or less to measure the enzymic rate of destruction. At the present time we have no evidence as to the cause for the virtual cessation of enzymic destruction

TABLE 1

CAROTENE LOSSES IN DRYING LEAVES IN LIGHT (6000 FOOT-CANDLES) AND IN DARK

Leaf condition	Illumination	Weight loss as percentage of original fresh weight	Leaf temperature (° C.)	Time of treatment	No. of samples	Percentage carotene loss ± standard error
Fresh	Light	50–62	40–42	20– 60 min.	6	13.1 ±2.45
Fresh	Light	70–78	40–42	45–180 min.	5	35.6 ±2.20
Fresh	Light	80–90	40–42	60–130 min.	3	51.9 ±4.20
Autoclaved	Light	70–75	40–42	50 min.	2	34.0 ±3.95
Autoclaved	Light	84–88	40–42	60–170 min.	5	53.3 ±2.74
Fresh	Dark	20–30	30	30– 65 min.	4	4.08±0.83
Fresh	Dark	40–60	30	120–180 min.	4	10.2 ±1.68
Fresh	Dark	80–90	30	18– 29 hr.	5	31.0 ±1.42
Autoclaved	Dark	84–90	30	2¼–29 hr.	5	1.06±0.58

after 40–50% of the carotene has been destroyed. This phenomenon has been observed (**13**, pp. 116, 118) with barley leaves but apparently does not occur in alfalfa (**10**). Curve *A* (fig. 1) shows how the carotene destruction in prefrozen leaves (incubated at 37° C.) varies with time. The curve is very similar to that for dehydrated leaves; this is construed to mean that enzyme action in prefrozen and in partially dehydrated leaves is the same. The fact that the curve for dehydrated leaves lies below that for prefrozen ones is probably due to lower enzymic activity in marginal cells which are too dry for optimal enzymic activity and in cells along veins where water content is too high for initiation, or maximum rate, of enzymic activity (fig. 3).

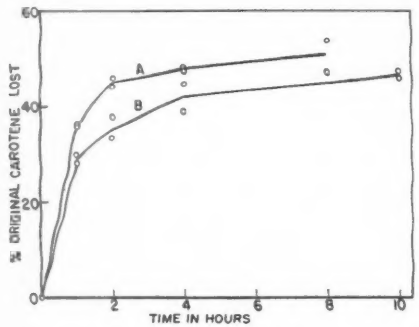

FIG. 1.—Effect of incubation period on amount of enzymic destruction of carotene. *A*, prefrozen leaves; *B*, predried leaves (85–91% of water removed). Incubation temperature, 37° C.

The influence of the time factor on the rate of photodestruction of carotenoids was studied with partially dehydrated leaves (fig. 2) and autoclaved leaves (fig. 9). Matched sets of leaves were dipped in mineral oil after pretreating (vacuum drying or autoclaving), one set then being exposed to light of a mercury vapor lamp, the other set serving as a dark control. Leaves which are partially dried (68–85% of water removed) before being exposed to light show an initially high rate of photodestruction and a decrease in rate of photodestruction at the point where further enzymic destruction practically ceases. After 24 hours these leaves still retained approximately 30 μg. of carotene (28%) and almost 100 μg. (55%) of carotenol per gram of original fresh weight. This incomplete destruction of carotenoids is probably due in part to incomplete killing of the leaf tissues. However, even in leaves completely killed by prefreezing, photodestruction of carotenoids is not carried to completion as rapidly as in autoclaved leaves. Figure 9 shows that, after only 6 hours, 80–90% of the carotene of autoclaved leaves is destroyed by the action of light alone. Autoclaving therefore renders the carotenoids susceptible to more complete photodestruction, a fact which has been observed by MITCHELL and HAUGE (**10**) and by JONES *et al.* (**7**). This effect makes comparison of carotene destruction in autoclaved and unautoclaved leaves in light invalid as a means of determining enzymic destruction by difference, since autoclaving does more than simply eliminate enzymic destruction of carotene. Thus GUILBERT's (**4**) work does not give a true estimate of the relative importance of enzymic and photodestruction of carotene.

From the data in figure 2, certain valid comparisons of the rates of photodestruction and enzymic destruction can be made. During the first 8 hours the leaves in the dark lost 28 μg., while the leaves in the light lost 63 μg. of carotene per gram of original fresh weight. This would mean 28 μg. lost by enzymic destruction and 35 μg. lost by photodestruction. This is a valid comparison only for the conditions of the experiment. If the leaves had been predried until 85–

90% of the water had been lost instead of only about 75%, then the enzymic destruction would have been higher and would have accounted for approximately 40% of the initial carotene content (fig. 1), and the photodestruction might have been somewhat less (fig. 4). Another factor which renders difficult the comparison of photodestruction and enzymic destruction is the fact that in bean leaves the rate of enzymic destruction falls off sharply after 40% of the carotene has been destroyed. Therefore, quite different results can be obtained by comparing enzymic and photodestruction after 2, 8, and 24 hours of exposure of the leaves. If the variability of light and temperature encountered in the field is considered, it becomes evident how difficult it would be to evaluate the importance of enzymic and photodestruction under field conditions. By following a procedure such as used in this work (predrying or prefreezing), however, it is possible to determine the effects of various factors on the rates of photodestruction and enzymic destruction. In other words, we have been able to separate and distinguish these processes at least under controlled conditions, so that further study of the individual processes is possible. Finally, the relative amounts of photodestruction and enzymic destruction observed in this and other experiments indicate strongly that under ordinary field conditions of sun-curing, both the enzyme- and the light-mediated destruction of carotenoids are of importance and must be considered in any program to improve retention of carotenoids in leaves.

MOISTURE CONTENT AND CAROTENE DESTRUCTION.—Having determined appropriate time intervals for the study of rates of carotene destruction, the influence of moisture content on the rate of enzymic carotene destruction was investigated. A number of leaf sets were dried *in vacuo* at room temperature to different moisture contents. Control sets were blanched at this point, and remaining samples were incubated in the dark for 1 hour at 37° C. The maximum enzymic destruction occurs when 80–95% of the water has been removed (fig. 3). Since the curve showing the rate of carotene destruction rises steeply when 75–82% of the water had been lost, death of the cell, or sufficient injury to cause accelerated carotene destruction, may be assumed to occur in this moisture range. That there are measurable carotene losses when 25% of the moisture had been removed is construed to mean that some of the marginal cells have lost 75%

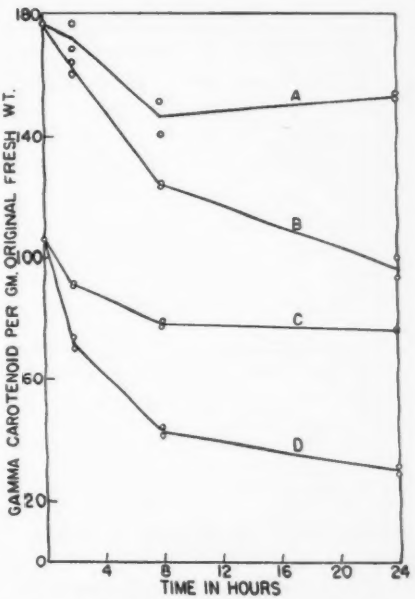

FIG. 2.—Carotenoid content of predried leaves (68–85% of water removed) incubated in dark and in light as a function of time. *A*, carotenol, dark; *B*, carotenol, light; *C*, carotene, dark; *D*, carotene, light. Light intensity, 1300 foot-candles; temperature, 23°–24° C.

or more of their moisture. The abrupt drop in rate of carotene destruction after 95% water loss means that the amount of water required for enzymic activity is very small. The fact that the rate of carotene destruction fell to zero when leaves were completely dry shows that some water is essential for enzymic activity. It should be pointed out that enzymic destruction of carotenoids is relatively little influenced by moisture content

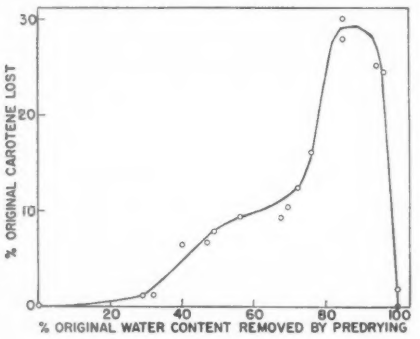

FIG. 3.—Effect of moisture content on enzymic destruction of carotene. Leaves predried *in vacuo* at room temperature before incubation at 37° C. Carotene loss calculated on basis of carotene remaining after dehydration. Incubation time, 1 hour.

(except in completely dry leaves) if the cells are prekilled by freezing.

The effect of the water content of bean leaves on photodestruction was studied with predried leaves prepared in the same way as described above. The leaves, after partial drying to desired water content, were dipped in mineral oil and spread out in open dishes. One set of each pair of matched sets was placed in the light for 3 hours, while controls were kept in the dark at the same temperature (24°–25° C.). The difference in carotene and carotenol content between dark controls and sets exposed to light is a measure of the amount of photodestruction which occurred (fig. 4). Replicate experiments were run in the morning and afternoon of the same day. Although the general shape of the curves is the same, indicating maximum photodestruction in leaves which had lost approximately 75% of their water in predrying, the absolute values for the samples harvested and run in the afternoon are considerably higher than those for the morning. This indicates a marked effect of physiological condition of the leaf, influenced possibly by previous exposure to light, on the stability of carotenoid pigments to photodestruction under the conditions employed.

Maximum photodestruction occurs at a higher water content (at 65–85% dehydration; i.e., 65–85% of water removed in predrying) than maximum enzymic destruction (approximately 80–95% dehydration). This is due in part to the decrease in leaf area with drying and consequent decrease in light absorption per unit of pigment. However, photodestruction decreases to a greater extent than can be accounted for by shrinkage of the leaf as it approaches complete dryness. Leaves which were 89% dehydrated lost 33% less carotene through photodestruction than did leaves 75% dehydrated. The decrease in leaf area, however, was only 18%. The discrepancy is even larger when leaves 100% dehydrated are compared with those which were 89% dehydrated. Here the decrease in leaf area was 19%, while the amount of photodestruction decreased 71%. It is clear that water, directly or indirectly through effects on factors other than leaf area, increases the rate of photodestruction in partially dried leaves in the region of 75–100% dehydration. However, some photodestruction does occur in leaves which are completely dry. The maximum rate of photodestruction with reference to water content, therefore, lies between that water loss which is necessary to kill

or injure the cell and the point at which further loss of water retards the photodestructive process. Since all the cells of the leaf do not dry out uniformly (the marginal cells becoming dry first), the shape of the curves in figure 4 can be explained as the balance between killing or injuring of progressively larger numbers of cells, with initiation of photodestruction in them, and complete drying-out of

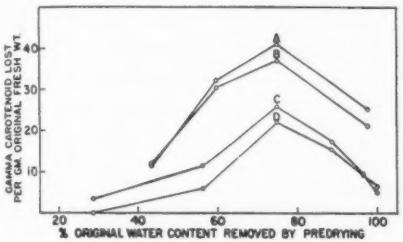

FIG. 4.—Effect of moisture content on photodestruction of carotenoids. Leaves predried *in vacuo* at room temperature before exposure. Carotenoid content of leaves in light subtracted from carotenoids in matched sets in dark to give amount of photodestruction. *A* and *B*, carotenol and carotene, respectively, for samples prepared from leaves picked at 2:00 P.M. *C* and *D*, carotene and carotenol, respectively, for samples prepared from leaves picked at 9:00 A.M. Light intensity, 1000 foot-candles; exposure period, 3 hours.

progressively larger numbers of cells, with concomitant decreases in rate of photodestruction in such cells.

TEMPERATURE AND CAROTENE DESTRUCTION.—Temperature characteristics for the enzymic oxidation of carotenoids are given in figure 5. Matched sets of fresh leaves and autoclaved leaves (as controls) were prefrozen, brought to the desired temperatures quickly by dipping in water slightly warmer than these temperatures, and incubated for 1 hour at the different temperatures. The maximum in the curve is a result of two opposing effects—the increase in rate of carotene destruction with higher temperature and the increase in rate of enzyme inactivation with rising temperature. For the time interval employed, the maximum enzymic destruction of carotene was obtained at approximately 37° C. The temperature coefficients for a 10° rise in temperature (Q_{10}), calculated for the ranges of 4°–15° C. and 15°–24° C., are 1.68 and 1.59, respectively. These values are reasonable for enzymic reactions (**15**). It should be noted that at 80° C. there was appreciable carotene destruction which indicates that the destruction of the enzyme even at this temperature is by no means instantaneous.

The effect of temperature on the rate of photodestruction of carotenoids was determined. Since in autoclaved leaves a wide temperature range can be used without fear of disrupting the system, rates of photodestruction were determined at only two widely separated

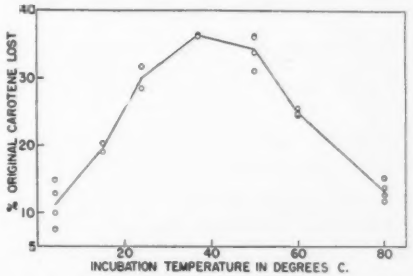

FIG. 5.—Effect of temperature on enzymic destruction of carotene in prefrozen leaves. Carotene loss calculated on basis of carotene content of blanched controls. Incubation time, 1 hour.

temperatures. Matched sets of leaves were exposed at 24° C. and 64° C. in light and in dark by floating the leaves on water maintained at these temperatures. Light intensities (mercury vapor lamp) of 200 foot-candles (light limiting) and 1400 foot-candles were used. In replicated experiments the Q_{10} for both light intensities calculated from observations at these two temperatures was 1.2–1.3. This

temperature coefficient falls within the range of coefficients for photochemical reactions in which temperature affects some nonphotochemical step in the reaction (3). Since the temperature coefficient of photodestruction is low, adequate control of temperature was easily obtained by performing experiments in a room in which fluctuations were limited to 1° or 2° C.

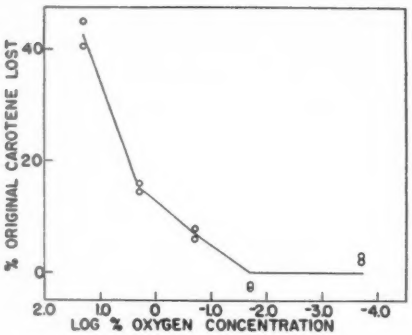

FIG. 6.—Effect of oxygen concentration on enzymic destruction of carotene in prefrozen leaves. Incubation time, 2½ hours; temperature, 37° C.

OXYGEN AND CAROTENE DESTRUCTION.—The relation of molecular oxygen to enzymic destruction of carotene was investigated. Matched leaf samples were placed in 8-oz. glass bottles. The oxygen concentration was adjusted by evacuating to a known pressure and restoring to atmospheric pressure with tank nitrogen scrubbed free of oxygen with alkaline pyrogallol. By repeating this process, the oxygen tension could be reduced to any desired value. Fresh leaf sets were frozen in the bottles after the desired oxygen concentration had been obtained. Control samples (autoclaved leaf sets) were treated exactly the same as the fresh leaves. After freezing, leaves were incubated at 37° C. for 2½ hours. Figure 6 shows that enzymic destruction of carotene is reduced at lower oxygen tensions and that it falls to zero in the region of 0.02–0.2% oxygen. This proves that enzymic destruction of carotene is oxidative and that molecular oxygen is necessary for the process. This is in interesting contradistinction to the photodestruction of carotene in which some destruction occurs in the absence of molecular oxygen (fig. 7). It would appear that the decolorization of carotene by substances other than oxygen is activated by light.

In studying oxygen tensions and photodestruction, leaf sets were placed in the bottles so that they could be subsequently exposed to light. They were then autoclaved, and the oxygen tension lowered to the desired values. The samples were exposed to 900 foot-candles for 2–4 hours, the time varying in different ex-

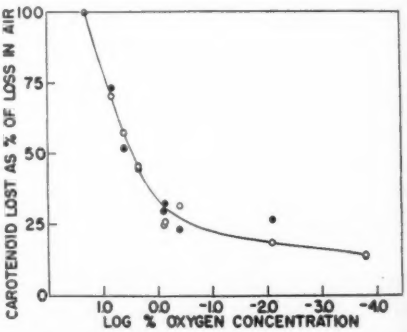

FIG. 7.—Effect of oxygen concentration on photodestruction of carotenoids in autoclaved leaves. *Open circles*, carotene; *closed circles*, carotenol. Light intensity, 900 foot-candles; temperature 23° C; exposure period, 3 hours.

periments. In a series of experiments (table 2) leaves in an atmosphere of nitrogen lost 23.9±3.5% as much carotene and 18.9±2.1% as much carotenol as the samples in air (means of five determinations). Figure 7 gives the results of a series of experiments in which intermediate oxygen tensions were employed. Between 20% and 0.5–1.0% oxygen, the photodestruction was proportional to the

logarithm of the oxygen concentration (11, p. 530 [on relation of oxygen tension to photo-oxidation and respiration]). Below 0.5% oxygen, photodestruction decreased only slightly with decreasing oxygen tensions. In several experiments (II and III, table 2) it was found that in nitrogen as much photodestruction occurred between 2 and 4 hours as occurred in the first 2 hours. If photodestruction of carotenoids in the absence of molecular oxygen is oxidative, some cellular constituents may function as oxidants in place of oxygen; it is also possible that photodestruction of carotenoids in absence of oxygen may proceed through reduction of carotenoids rather than oxidation (11, p. 505). However, under such conditions, the rate of photodestruction is only about 20% of what it is in air.

TABLE 2

PHOTODESTRUCTION OF CAROTENOID PIGMENTS IN AUTOCLAVED LEAVES IN AIR AND IN N_2. CAROTENOIDS DESTROYED IN LIGHT EXPRESSED AS PERCENTAGE OF CAROTENOID CONTENT OF DARK CONTROLS

Experiment	Atmosphere	Light intensity (foot-candles)	Duration (hours)	% pigment lost		Loss in N_2 as percentage of loss in air	
				Carotene	Carotenol	Carotene	Carotenol
I	Air	1400	3	76.5	53.0		
	N_2	1400	3	19.0	10.3	24.9	19.4
II	Air	1000	2	48.0	31.0		
	N_2	1000	2	11.5	6.4	24.0	20.6
	Air	1000	4	80.0	62.4		
	N_2	1000	4	27.8	16.0	34.8	25.7
III	Air	1000	2	44.1	28.8		
	N_2	1000	2	5.6	4.2	12.7	14.6
	Air	1000	4	58.9	38.4		
	N_2	1000	4	13.6	5.4	23.1	14.1
Means ± S.E.						23.9±3.5	18.9±2.1

Light intensity, light quality, and photodestruction.—The relationship of light intensity to photodestruction was investigated with autoclaved leaves early in the course of these studies. Leaves were floated on water and exposed for 6 hours to different intensities of light by varying the distance from the light source (mercury vapor lamp). Control sets were analyzed after autoclaving. The results (fig. 8) indicate that up to about 300 foot-candles (measured by Weston meter), photodestruction is roughly proportional to light intensity. Increasing the light intensity above 300 foot-candles results in increased photodestruction but not in the same proportion observed below 300 foot-candles. In most of the work on photodestruction, light intensities of 1000–1500 foot-candles have been used, since an appreciable amount of photodestruction will occur in a few hours at these intensities.

The effect of light quality on photodestruction was determined by interposing dye solutions between the light source (a 1000-watt tungsten-filament lamp) and the autoclaved leaf samples. A primary filter of copper acetate solu-

tion was used to filter out the infrared radiation and reduce the proportion of energy in the red end of the spectrum. Transmittancies of the dye solutions and copper acetate were determined with a Beckman spectrophotometer (model 11, 1-cm. cells) and are given in figure 10. The yellow-green filter transmitted some light in the blue-violet region, but this contamination is unimportant, since the

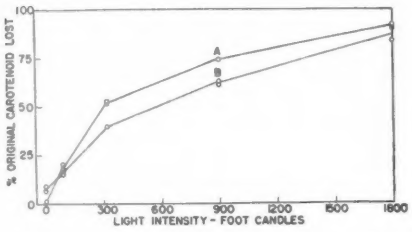

FIG. 8.—Light intensity and photodestruction in autoclaved leaves. Matched sets analyzed for original carotenoid content, after autoclaving. *A*, carotene; *B*, carotenol. Duration of experiment, 6 hours; temperature, 23°–25° C.

output of the light source used in the experiment was so low in the blue-violet region. By varying the concentration of the dyes, the light energy transmitted by each filter was equalized. Light energy was measured with a calibrated thermopile and was 100 or 150 μ watts/sq. cm., varying in different experiments. By spectroscopic observation (Hilger spectrometer) of transmitted light from the light source, the bands reported in table 3 were visible.

The results (table 3) show that photodestruction occurred in the orange and yellow-green regions of the spectrum as well as in the blue. Since carotenoid pigments do not absorb light in the yellow-green or orange regions, it seemed likely that the light was being absorbed by chlorophyll, which then directly or indirectly caused the oxidation of the carotenoids.[1] This view was supported by the fact that photodestruction by yellow-green light was somewhat less than that caused by blue or orange light. An experiment (III, table 3) was then run with autoclaved, etiolated bean leaves, which, by measurement of light absorption of extracts at 670 mμ, were shown to contain only about 1–2% as much chlorophyll as normal bean leaves. In this case, owing to the low initial carotene content of the leaves, the carotenol data only are reported. Despite the low chlorophyll content of the leaves, photodestruction of carotenol occurred in all three regions of the spectrum. The original carotenol content was approximately 50% as high as in green leaves. Photodestruction of carotenols (experiment II, table 3) amounted to about 10%. Allowing for the longer time interval used in experi-

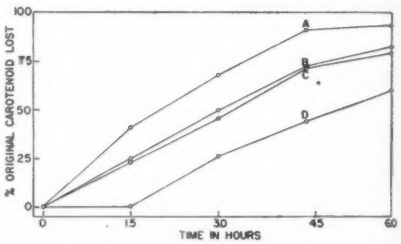

FIG. 9.—Effect of leaching autoclaved leaves on subsequent rates of photodestruction of carotenoids. Difference between carotenoid content of dark controls and light-treated sets expressed as percentage of carotenoids in controls. *A*, carotene loss in leached leaves; *B*, carotene loss in unleached leaves; *C*, carotenol loss in leached leaves; *D*, carotenol loss in unleached leaves. Light intensity, 1200 foot-candles; temperature, 23°–24° C.

ment III, the amount of carotenol destroyed, on an absolute basis, compares fairly well with the amount of photodestruction in experiment II. It would seem, therefore, that if chlorophyll is the light-absorbing agency in the photodestruction of carotenoids, much lower

[1] Cf. FRANCK and FRENCH (2) on photautoxidation in blue and red light.

concentrations of chlorophyll than normally occur in green leaves are effective in promoting photodestruction.

EFFECT OF LEACHING WITH WATER ON PHOTODESTRUCTION.—From early experiments on the effect of the time factor on photodestruction, it was suspected that natural antioxidants in the leaf were retarding photodestruction of the pigments. An experiment was run in which

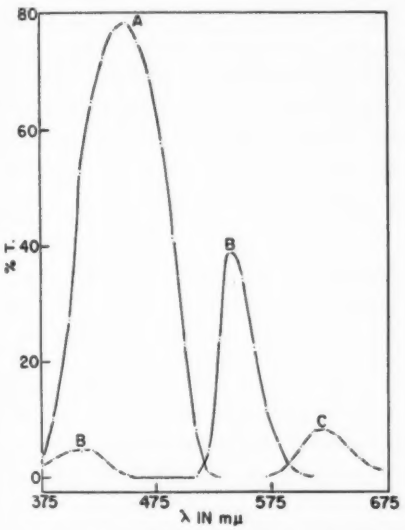

FIG. 10.—Transmittance of dyes used in study on effect of light quality on photodestruction, determined by Beckman spectrophotometer. Results calculated for combined absorption by primary filter (1 cm. 2% copper acetate) and 1 cm. of following dye combinations: *A*, 0.01% Pontamine Fast Turquoise 8GL and 0.005% Victoria Pure Blue BO; *B*, 0.01% Pontamine Fast Turquoise and 0.01% Orange G (Du Pont); *C*, 0.02% Orange G and 0.02% Fast Red (Coleman-Bell).

the photodestruction in leaves blanched by boiling water for 5 minutes (leached material) was compared with that in leaves blanched by steam (unleached material). The photodestruction of carotene after 1½ hours was almost twice as high in the leached material as in the unleached. A time study was made in which half the samples were blanched dry in the autoclave and the other half blanched in preheated water in the autoclave. The latter were then soaked for several hours in cold water to assure complete leaching of water-soluble constituents. The leaf sets, covered with mineral oil, were exposed to light, with controls kept in the dark. The results show (fig. 9) that, during the first 1½ hours, the carotene loss in leached leaves was 60% higher than in unleached leaves. Carotenols were not photodestroyed at all in unleached leaves during this period, while appreciable photodestruction occurred in leached leaves. After the first 1½ hours, the rates of photodestruction were the same in leached and in unleached leaves. A definite induction period owing to the antioxidant action of water-soluble constituents of the leaf has been demonstrated.[2]

TABLE 3

PHOTODESTRUCTION OF CAROTENOID PIGMENTS BY LIGHT OF DIFFERENT SPECTRAL REGIONS

SPECTRAL REGION IN Mμ	% CAROTENE DESTROYED IN GREEN LEAVES		% CAROTENOL DESTROYED IN ETIOLATED LEAVES
	Experiment I*	Experiment II*	Experiment III*
430–505	16.9	16.7	43.6
520–590	15.1	14.1	33.2
600–640	17.5	20.0	45.4

* Conditions of experiments: I, duration, 6 hours; light energy, 150 μ watts/sq. cm.; II, duration, 9 hours; light energy, 100 μ watts/sq. cm.; III, duration, 18 hours; light energy, 100 μ watts/sq. cm.

Discussion

The marked effect of the water content of partially dried leaves on the en-

[2] Cf. RABINOWITCH (11, p. 526) on photo-oxidation of cellular oxidation substrates in preference to oxidation of pigments.

zymic destruction of carotene has been demonstrated (fig. 3). Bean leaves which were 85% dehydrated lost carotene at almost twice the rate of loss observed in leaves which were 75% dehydrated. As complete dryness is approached, the rate of change in enzyme activity is even more pronounced, with completely dry leaves showing no enzymic destruction of carotene during incubation. We have referred previously to the experiment of MITCHELL and HAUGE (10), in which carotene loss in leaves drying in the dark is used as a measure of enzymic destruction in leaves drying in the light. From the above considerations, it can be seen that small differences in rates of drying may result in different amounts of enzymic destruction in the light and in the dark, giving erroneous results in the calculated amount of photodestruction.

Furthermore, small fluctuations in the rate of drying of leaves exposed to similar conditions of light and temperature may result in considerable variations in the relative importance of enzymic destruction and photodestruction because of the differential effect of moisture content on these two processes (figs. 3, 4). For example, leaves which have lost 75% of their water will have a maximum rate of photodestruction of carotenoids, while leaves which have lost 85% of their water will exhibit a maximum rate of enzymic destruction. The relative periods of time that leaves remain at these moisture contents will influence the relative importance of these two processes.

The influence of light on leaf temperature may further invalidate such studies. In the experiment reported in table 1, the observed temperature of leaves in light was approximately 10° C. above air temperature and leaf temperature in the dark. The higher leaf temperature in the light would influence enzymic destruction through the effect of temperature per se on enzymic activity and the effect of temperature on the rate of drying. In the present experiments with photodestruction we were able to limit differences in leaf temperature in light and in dark to 1° C., even with light intensities approaching 2000 foot-candles, by using a water filter to reduce infrared radiation and to cool the leaves by reradiation to the cold water.

In our work with bean leaves the duration of exposure of leaves to conditions favoring photodestruction and/or enzymic destruction is a critical factor. The rate of enzymic destruction is not constant, being initially high, and decreasing to virtually zero after some 40–50% of the carotene has been destroyed (fig. 1). Although the photodestructive rate is more regular in autoclaved leaves (fig. 9), it also decreases markedly with time (fig. 2) in unautoclaved (predried) leaves. Photodestruction, however, continues at an appreciable rate after enzymic destruction virtually ceases. The time interval used in comparing relative rates of photodestruction and enzymic destruction will therefore greatly influence the results.

From the data in figure 8, the dependence of rate of photodestruction of carotenoids on light intensity is obvious. Any estimation of the importance of photodestruction would be influenced by the light intensity incident on the exposed leaves. In terms of field practice, the greater retention of carotene in hay dried in the windrow than in the swath (17) is an effect attributable, in part at least, to the lower light intensity incident on much of the hay in the windrow.

Our study of the factors influencing photodestruction and enzymic destruction of carotene has made it possible to measure these processes accurately in

material which, although under controlled environmental conditions, is directly comparable with material under field conditions. By predrying leaves in a vacuum to a given moisture content and maintaining them at such moisture content during the period of a test, we are able to arrest the complex, ever changing conditions which occur in the field and so to study the effectiveness of various environmental and physiological factors on the rate of destruction of the carotenoid pigments. We have further shown that prefreezing is a useful technique for such studies, since material which is more uniform in regard to the condition of the leaf cells is obtained, while the processes of carotene destruction are qualitatively unaltered by the freezing treatment.

The probability that the carotene-destroying processes are indirect improves the likelihood of success in controlling them and in improving the retention of the carotene in field-cured hay crops. MITCHELL and HAUGE (9) have presented evidence that the enzyme involved in the destruction of carotene in leaves may be a lipoxidase. The coupled oxidation of carotene (14, 16) by the lipoxidase-fat system offers greater opportunities for control than if carotene were the direct substrate of enzymic action. Similarly, the fact that light does not act directly on carotene to cause photodestruction improves the possibility of protecting the carotene.

The presence of naturally occurring water-soluble substances which function as antioxidants for the photodestruction of carotene in bean leaves is of interest in several connections. First, the determination of the character of these substances would be of value per se. Second, the observation that bean leaves may differ in their susceptibility to photodestruction, depending on the time of day the leaves are picked from the plants (fig. 4), may be due to differences in the natural antioxidant content associated with time of day. Hence, in any study on the effect of physiological and environmental conditions on photodestruction and enzymic destruction of carotenoids, due consideration must be given to possible variations in the quantity of natural antioxidants.

Summary

1. The enzymic and photodestructive processes affecting the carotene content of bean leaves have been studied. By pretreating leaves (drying or freezing) and controlling the conditions of experimental exposure, we have been able to measure quantitatively the separate processes of carotene destruction in leaves in which both enzymic destruction and photodestruction have occurred simultaneously.

2. In partially dried leaves, carotene is destroyed rapidly both by enzyme- and by light-mediated processes until about 50% of the carotene is gone. The rates of destruction, especially the enzymic rate, then decrease, although in the light a further gradual decrease in carotene content has been observed. In autoclaved leaves, however, photodestruction continues at a high rate until 80–90% of the carotene is destroyed. In prefrozen leaves, incubated at 37° C. in the dark, 45% of the carotene is destroyed by enzymic action in 2 hours, but the remaining carotene is stable. Our results indicate that both the photodestructive and the enzymic processes are important in accounting for the losses of carotene which occur in field-curing of hay.

3. As a leaf dries out, the cells in the margin of the leaf suffer injury or death first, and carotene destruction is initi-

ated in progressively increasing numbers of cells. In tests of the enzymic destruction of carotenoids in leaves held at given moisture contents, maximum destruction of the pigments was found in leaves which had lost 80–95% of their water by predrying. In completely dehydrated leaves, no enzymic destruction of carotenoid pigments occurred. Photodestruction also becomes progressively greater in leaves which have decreasing moisture contents and reaches a maximum when about 68–80% of the water had been lost by predrying. Completely dehydrated leaves show a low but distinct rate of photodestruction.

4. Enzymic destruction, in the time interval used, is at a maximum at approximately 37° C. and decreases at higher temperatures. The temperature coefficient (Q_{10}) for enzymic destruction is 1.6–1.7 between 4° and 25° C., and is 1.2–1.3 for the photodestructive process between 24° and 64° C.

5. Atmospheric oxygen is essential for enzymic destruction of carotenoids. In an atmosphere containing 0.02% oxygen or less, enzymic destruction does not occur. Photodestruction is also influenced by atmospheric oxygen, and the rate of photodestruction is proportional to the logarithm of oxygen concentration from about 0.5 to 20% oxygen. Below 0.5% oxygen, further decreases in oxygen tension do not appear to influence photodestruction appreciably. It is suggested that some cellular constituents can replace atmospheric oxygen in the photodestruction of the carotenoid pigments or that the carotenoids are destroyed by photoreduction in the absence of oxygen.

6. Photodestruction of carotenoids is roughly proportional to light intensity up to approximately 300 foot-candles. Although photodestruction continues to increase at higher light intensities, the relationship is not the same as at low light intensities. Photodestruction has been shown to occur equally well in orange, yellow-green, and blue light, proving that the light energy is not absorbed directly and exclusively by the carotene. The influence of chlorophyll content has been investigated. The photodestruction of carotenols was found to be as great in etiolated leaves (having only 1–2% of the chlorophyll content of normal green leaves) as in green leaves. Furthermore, photodestruction occurred in orange light as well as in blue light in the etiolated leaves.

7. Naturally occurring antioxidant(s) have been demonstrated in bean leaves. An induction period is noted in the photodestruction of carotenols.

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SELECTION AND BREEDING FOR HIGH β-CAROTENE CONTENT (PROVITAMIN A) IN TOMATO[1]

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Introduction

In the summer of 1942 a survey for β-carotene content (provitamin A) was made of 240 varieties, wild types, and hybrids of four species of tomato (*Lycopersicon*). The results, showing very high β-carotene content in certain hybrids, were reported in 1943 (1). Subsequent work has been directed toward genetic stabilization of hybrid material with high β-carotene content and the development of corresponding commercial-type varieties. The results of these efforts are reported in this paper.

Methods

Fruits were grown, sampled, and analyzed as described in detail in a previous publication (2). In brief, representative fruits were homogenized in a Waring blendor, and a weighed aliquot was extracted with acetone and hexane (75:60). Acetone was removed by washing with water. Xanthophylls and esters were removed with 90% methanol and 20% KOH in methanol. Spectroscopic readings (for β-carotene and lycopene determinations) were made directly on the resultant hexane solution. Check values for β-carotene content were obtained by chromatographic separation and spectroscopic analysis.

[1] Journal paper no. 286 of the Purdue University Agricultural Experiment Station. This investigation was supported in part by a grant from the Nutrition Foundation, Inc., and is a joint contribution of the departments of botany and plant pathology and of agricultural chemistry.

[2] Deceased, formerly Assistant Geneticist; [3] formerly Associate Chemist; [4] formerly Technical Assistant.

Results

Discovery and selection of high β-carotene lines

The survey in 1942 of red- and yellow-fruited species of *Lycopersicon* did not reveal any selections remarkably high in β-carotene content. In this survey the 156 red- or yellow-fruited selections, representing the species *L. esculentum* and *L. pimpinellifolium*, possessed a β-carotene concentration ranging from 1.0 to 19.2 γ/gm. and a lycopene content ranging from 0.8 to 463.0 γ/gm. on the fresh-weight basis.[5] The 23 commercial varieties included in the above selections ranged near the mean of all observations. As determined at that time and in later studies, the green-fruited species *L. peruvianum* and *L. hirsutum* were very low in carotenoids, ranging from 0.7 to 3.6 γ/gm. of β-carotene and from zero to 1.4 γ/gm. of lycopene.

Although the survey of the species and varieties revealed a few selections of promise as parental material in breeding for high β-carotene content, a much better selection was obtained from the backcross [Baltimore × F_1 (Rutgers × *L. hirsutum* P.I. 126445)]. The latter contained three and a half times as much β-carotene (67.5 γ/gm.) as the highest of the red- or yellow-fruited selections. Also, it contained nine times as much β-carotene as the highest, and thirteen times as much as the average, of the commercial varieties.

Selection 4079-5012 is a single-plant selection from the F_3 population [Indiana Baltimore × F_1 (Rutgers × *L. hirsutum* 126445)] that has been used extensively as a high β-carotene parent. The original selection is small-fruited and of poor quality and low yield. The fruit size and carotene content of this selection are given in table 1. The total carotene fraction includes lycopene. Included in the same table are the results of further selection in later generations for high β-carotene content. Of eighteen progeny plants analyzed in the F_4 generation in 1943, plant no. 9 contained the highest content of β-carotene. F_5 and F_6 progenies derived from this plant also contained high concentrations of β-carotene. The selection within line 4079-5012 indicated that the factors primarily responsible for high β-carotene content were fixed relatively early in the selection process, but apparently factors with minor influences on the β-carotene content may still be heterozygous.

Transfer of high β-carotene to commercial types in the breeding program

When the selection 4079-5012 was used in crosses with Baltimore, Indiana Canners strain (cross 4360), fruits of the F_1 plants were intermediate or low in percentage and content of β-carotene (table 2). In the F_2, however, high β-carotene selections were recovered. The frequency with which high β-carotene lines were obtained from crosses suggests that the number of major factors necessary to obtain high β-carotene content, in addition to those present in Indiana Baltimore, is small. In the F_3 generation of cross 4360, in which selections were made only for the largest-fruited types, the recovery of high β-carotene lines was not so great as expected from the prior work on selecting within line 4079-5012. However, β-carotene has been increased to 83 γ/gm. in selections with fruit size equal to that of many commercial varieties but somewhat smaller than that of the recurrent commercial parents used in this program.

[5] Gamma (γ) is equivalent to microgram.

A second selection, 4079-5016, has proved better than 4079-5012 in transmitting fruit size to its progenies. This selection has been used extensively in crosses with Rutgers variety. Data are presented in table 3 on the fruit size and β-carotene content of selection 4079-5016, of Rutgers, and of selections within the F_1 and F_2 generation of crosses between Rutgers and 4079-5016. It is apparent that fruit size in cross A4408 is equal to Rutgers in many selections and that β-carotene content averages about twelvefold that of the Rutgers variety.

TABLE 1

β-CAROTENE CONTENT OF FRUITS IN ADVANCED GENERATIONS OF SELECTION 4079-5012

YEAR	CROSS	GENERATION	FRUIT WEIGHT (GM.)	CAROTENE FRACTION		
				Total (γ/gm.)	β-carotene	
					%	γ/gm.
1942	4079-5012*†	F_3	16	66	73	49
1943	4079-5012- 1*†	F_4	22	48	83	40
	3		24	71	13	10
	5		30	94	14	13
	7		24	64	15	10
	9*		15	85	93	79
	11		51	59	62	37
	13		33	82	34	27
	15		84	82	21	17
	17		24	56	92	51
	Mean of 18		28	63	55	35
1944	4079-5012-9- 1†	F_5	21	61	92	56
	3		22	110	97	107
	5		27	76	98	74
	7		15	83	94	78
	9		21	115	96	107
	11		28	71	96	69
	13		35	120	96	115
	15		20	90	97	87
	17		20	101	97	98
	19		22	87	96	83
	21		13	58	93	54
	23		24	100	96	96
	25		24	81	97	79
	Mean of 25		23	88	96	84
1945	4079-5012-9-9-×†					
	Mean of 12	F_6	23	101	88	89
	4079-5012-9-11-×†					
	Mean of 10	F_6	29	83	94	77
	4079-5012-9-13-×†					
	Mean of 12	F_6	34	92	82	79

* Indicates average value of more than one analysis.

† Parentage: [Baltimore × F_1 (Rutgers × *L. hirsutum* P.I. 126445)].

TABLE 2

β-CAROTENE CONTENT OF FRUITS OF BALTIMORE AND OF F_1, F_2, AND F_3 GENERATIONS OF CROSS OF BALTIMORE WITH HIGH β-CAROTENE SELECTION

YEAR	CROSS	GENERATION	FRUIT WEIGHT (GM.)	CAROTENE FRACTION		
				Total (γ/gm.)	β-carotene	
					%	γ/gm.
1942	Baltimore (mean of 5)	P	179	63	9	6
1943	Baltimore (mean of 17)	P	136	64	9	6
1945	Baltimore (mean of 9)	P	215	80	4	3
1943	4360– 2*	F_1	130	28	70	20
	4		55	67	9	6
	6		35	66	35	23
	8		188	101	6	7
	10		54	34	58	19
	12		52	45	50	22
	14		44	65	11	8
	16		21	86	59	50
	Mean of 16		68	63	40	22
1944	4360–16– 3*	F_2	70	95	96	91
	6		92	81	91	73
	9		70	101	92	92
	12		74	56	46	26
	15		35	56	71	39
	18		88	70	44	31
	23		86	90	40	36
	27		84	88	32	28
	30		48	55	40	22
	33		47	78	53	41
	36		137	54	28	15
	39		37	43	71	30
	42		39	51	49	45
	45		39	53	44	23
	46		142	58	87	50
	48		26	77	36	28
	51		61	61	25	16
	54		44	113	5	6
	57		72	72	44	32
	62		69	87	36	31
	65		44	98	84	82
	Mean of 65		62	75	52	39
1945	4360–16–3–1*	F_3	131	90	45	46
	2		159	88	46	41
	3		57	109	94	103
	4		116	99	10	10
	4360–16–6–1*	F_3	90	112	21	14
	2		194	39	24	17
	3		179	52	90	46
	4		125	113	45	49
	5		64	74	83	61
	6		119	104	80	83
	7		141	88	18	16
	8		129	75	37	28
	4360–16–45–1*	F_3	107	70	23	9
	2		126	69	54	37
	3		185	74	41	22
	4		95	116	21	25
	4360–16–62–1*	F_3	125	81	28	23
	3		77	85	20	17
	5		111	104	9	9
	7		122	87	30	26
	9		70	118	14	16
	Mean of 10		115	82	22	17

* Parentage of cross 4360: Baltimore × F_3 selection 4079–5012. 4079-5012 = [Baltimore × F_1 (Rutgers × *L. hirsutum* P.I. 126445)].

INTERRELATIONSHIP OF β-CAROTENE, LYCOPENE, AND COLOR

In the selections of high β-carotene content the mean concentrations of the total carotenes, calculated as the sum of the concentration of lycopene and β-carotene, have not been increased over that of Rutgers and Baltimore (tables 2, 3). It appears that the increased concentration of β-carotene has occurred at the expense of lycopene—the carotene primarily responsible for the red fruit color

TABLE 3

β-CAROTENE CONTENT OF FRUITS OF RUTGERS, OF SELECTION 4079-5016, AND OF F_1 AND F_2 GENERATON OF CROSSES BETWEEN THESE TWO PARENTS

YEAR	CROSS	GENERATION	FRUIT WEIGHT (GM.)	CAROTENE FRACTION		
				Total (γ/gm.)	β-carotene	
					%	γ/gm.
1942	Rutgers	P	255	56	7	4
1943	Rutgers (mean of 5)	P	125	60	5	3
1944	Rutgers (mean of 3)	P	235	77	3	2
1945	Rutgers	P	252	98	< 1	< 1
1942	4079–5016*	F_3	66	45	54	26
1943	4079-5016–2*	F_4	122	27	93	25
1944	4408–1*†	F_1	160	45	11	5
	2		77	78	47	37
	3		122	94	9	8
	4		171	53	41	22
	5		91	77	37	29
	6		154	53	12	6
	7		138	91	8	7
	A4408–1†	F_1	123	73	46	34
	2		88	85	9	8
	3		138	103	3	3
	4		151	86	12	11
	5		111	114	2	2
1945	4408–2–×†(mean of 29)	F_2	111	83	37	23
	4408–4–×†(mean of 8)	F_2	213	77	33	26
	4408–5–×†(mean of 46)	F_2	147	86	27	22
	A4408–1– 2†	F_2	132	53	72	38
	4		177	81	81	66
	6		176	75	54	40
	8		206	83	61	56
	10		214	104	< 1	< 1
	12		148	79	4	3
	14		141	107	8	8
	16		187	70	32	20
	18		167	72	45	32
	20		242	96	36	35
	22		202	77	87	66
	24		165	69	46	32
	26		134	82	38	31
	28		133	118	2	2
	30		264	147	10	15
	32		220	88	44	40
	34		134	92	18	17
	36		165	63	37	42
	38		121	82	5	4
	Mean of 38		180	81	39	39

* Parentage: [Baltimore × F_1 (Rutgers × *L. hirsutum* P.I. 126445)].

† Parentage: Rutgers × F_4 selection of 4079-5016–2.

in most varieties of tomatoes. Consequently, fruits containing a high concentration of β-carotene are of deep orange color. However, analyses of orange and orange-yellow varieties currently on the market, such as Jubilee and Tangerine, have shown them to be no higher in β-carotene content than the standard red-fruited varieties. Their orange color is due largely to isomers of lycopene which do not have vitamin A activity.

β-carotene per gram of fresh fruit have been obtained.

Relative value of tomatoes as a source of provitamin A in human diet

The β-carotene content of several vegetables commonly recommended as excellent sources of provitamin A in the human diet is given in table 4. As a source of provitamin A, tomatoes selected for high β-carotene content are

TABLE 4

β-Carotene content of some vegetables recommended as excellent sources of provitamin A

Vegetable	β-Carotene (γ/gm.)	
	Fresh	Dry
Carrot*	90	900†
Spinach*	54	.....
Sweet potato*	44	120†
Squash, winter*	7	.....
Tomato, commercial*	7	120‡
Tomato, high β-carotene selection	83	1200

* Values calculated from data presented in *Tables of Food Composition*, published by Committee on Food Composition, National Research Council.

† Values on dehydrated product.

‡ Assume approximately 6% dry matter.

All fruits analyzed in the breeding project have been classified for color. Generally, when the β-carotene content is greater than 35% of the total carotene pigments present, the fruit is classified as orange. Fruits of characteristic tomato redness are relatively low in β-carotene content, at least 65% of the carotenes present being lycopene. Because of present consumer demands for red tomatoes, work is being directed toward developing commercial varieties not only of the highest possible β-carotene content (orange-colored) but also of the highest possible β-carotene content compatible with red fruit color. At the present time, red selections containing approximately 30 γ of about equal to carrots and will surpass most other foods on a fresh-weight basis. When determined on the dry-weight basis, the superiority of these high β-carotene selections as a source of provitamin A in the diet becomes even more marked.

Summary

1. High β-carotene concentration in tomato fruits has been obtained from selections of the cross [Baltimore × F_1 (Rutgers × *L. hirsutum* P.I. 126445)]. One selection from this backcross generation produced an average content of 101 γ/gm. of crude carotene, of which 88% occurred as β-carotene.

2. Large-fruited, high β-carotene selections have been obtained, by further backcrossing to commercial varieties, at a frequency great enough to suggest that the number of major factors necessary to obtain high β-carotene, in addition to those present in commercial varieties, is small.

3. β-Carotene appears to be produced at the expense of lycopene, since the total carotenoid concentration in the high β-carotene selection is not increased over that of the red, low β-carotene parent.

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TEMPERATURE COEFFICIENTS OF CELL ENLARGEMENT[1]

MARIAN DELLERS CHAO AND W. E. LOOMIS

Introduction

Cell enlargement has been generally regarded as a relatively simple process involving little or no synthesis of protoplasm, with the increase of cell volume resulting from absorption of water through osmotic or imbibitional forces. THUT and LOOMIS (9), however, observed that the growth rates of several plants showed the temperature coefficients of chemical reactions. Even the final growth of leaves of *Ricinus communis* nearly 40 cm. wide showed a temperature coefficient of 2.0 or more, and this in spite of the bright sunlight and lower daytime humidity which accompanied the higher temperatures. Osmosis, and at least certain types of imbibition, acting alone would show the temperature coefficients of physical reactions with Q_{10} values just above 1.0, where

$$Q_{10} = \frac{\text{Rate at } t + 10^\circ \text{ C.}}{\text{Rate at } t^\circ \text{ C.}}.$$

Q_{10} values of 2.0 or more are taken as evidence that chemical reactions of some sort are limiting for the over-all process.

The data presented here support the thesis that the enlargement of individual cells, or the enlargement of organs in which cell number is no longer increasing, is limited by chemical processes, either in the protoplasm, in the cellular membrane, or in the cell wall.

Experimentation

DANDELION SCAPES.—Excised sections of young scapes of *Taraxacum officinale*, taken from directly beneath small buds, were grown in nonsterile solutions of sucrose and indoleacetic acid (IAA). The solutions were allowed to reach the experimental temperatures before the sections were inserted. Whole scapes, together with the attached buds, were brought into a cold room for preparation. Sections 1 cm. long from each of ten scapes were cut and immediately placed in 25 ml. of solution in a Petri dish at the experimental temperature. Solutions

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were changed daily, and bacterial growth was very slight, the solutions remaining clear and no tissue degeneration becoming apparent within 4-day test periods. Temperature coefficients were calculated for cell elongation. Coefficients for intervals other than 10° were obtained with the formula

$$Q_{10} = Q_{nc} = (Q_n)^c,$$

where n is the actual temperature interval studied and c is equal to $10/n$.

TABLE 1

ELONGATION OF EXCISED, 1-CM. SECTIONS AND OF EPIDERMAL CELLS OF DANDELION SCAPE DURING 96 HOURS

TEMPERATURE (° C.)	1% SUCROSE + 1.5 MG. IAA/LITER		DISTILLED WATER	
	Increase in section length	Increase in epidermal cell length	Increase in section length	Increase in epidermal cell length
0.....	1.18×*	1.02×	1.03×	1.01×
5.....	1.22×	1.27×	1.08×	0.99×
10.....	1.31×	1.17×	1.23×	1.11×
15.....	1.90×	1.56×	1.26×	1.24×
20.....	2.10×	1.83×	1.42×	1.37×
25.....	2.20×	1.90×	1.40×	1.22×
30.....	2.25×	1.71×	1.44×	1.11×

* Each figure is average of ten sections or of eighty cells.

Growth studies had shown that such sections of scapes would elongate two to three times in 4 days at room temperature in these solutions, with concentrations of indoleacetic acid ranging from 1 to 10 mg./l. and sucrose present in 1% concentration. As much as 50% elongation occurred, even in distilled water. The elongation of sections and of epidermal cells of sections of scapes after 96 hours in IAA-sucrose solution and in distilled water at various temperatures is shown in table 1. Even cooled sections placed in a pre-chilled solution at 0° C. showed a small but definite elongation.

In IAA-sucrose solution it is doubtful if any cell division occurred at 0° or 5° C. At temperatures from 10° C. through 30° C., however, the average increase in epidermal cell length was less than the increase in section length, and it is probable that some cell division was occurring. Since the ratio of the increase of epidermal cell length to the increase of section length remained nearly constant within this temperature range, however, higher temperatures apparently increased the rate of cell elongation to the same extent that they increased the rate of cell division.

The temperature coefficients of elongation of the sections and of the epidermal cells in IAA-sucrose solution during the 96-hour test period are shown in table 2. From 0° to 20° C., these Q_{10} values ranged from 1.7 to 4.1 for section and from 2.1 to 8.5 for cell elongation. The coefficients for cells are based on increase in average cell length as obtained by direct measurements of eighty cells in each lot with an eyepiece micrometer. They are thus minimum values, since any cell division would decrease the average cell length. These high temperature coefficients indicate that chemical reactions of some type

TABLE 2

TEMPERATURE COEFFICIENTS OF ELONGATION OF EXCISED SECTIONS AND OF EPIDERMAL CELLS OF DANDELION SCAPE

TEMPERATURE (° C.)	1% SUCROSE + 1.5 MG. IAA/LITER		DISTILLED WATER	
	Q_{10} for sections	Q_{10} for cells	Q_{10} for sections	Q_{10} for cells
0–10.....	1.7	8.5	7.7	11.0
5–15.....	4.1	2.1	3.2	24.0
10–20.....	3.5	4.9	1.8	3.4
15–25.....	1.3	1.6	1.5	0.9
20–30.....	1.1	0.9	1.0	0.3

were limiting influences in the elongation of cells of the scape.

In distilled water scape sections showed a smaller but unmistakable elongation (table 1). It is not clear whether cell division occurred during the 4-day growth period in water, although at temperatures of 25° C. and 30° C. the data suggest that it did. In general, the elongation in distilled water was much less than in the IAA-sucrose solution, and it is more difficult to distinguish the effects of temperature from the natural variation in the scapes. Nevertheless, high temperature coefficients of cell elongation were found from 0° to 20° C. (table 2), even in the absence of an external source of sucrose and auxin.

TABLE 3

ELONGATION OF EXCISED, 1-CM. SECTIONS AND OF EPIDERMAL CELLS OF DANDELION SCAPE IN 1% SUCROSE SOLUTION CONTAINING 5 MG. IAA PER LITER

TEMPERATURE (° C.)	24-HOUR PERIOD		36-HOUR PERIOD	
	Increase in section length	Increase in cell length	Increase in section length	Increase in cell length
0.5.....	1.10×*	1.38×	1.12×	1.00×
5.0.....	1.13×	1.32×	1.25×	1.02×
10.0.....	1.27×	1.14×	1.26×	1.26×
14.0.....	1.50×	1.66×	1.50×	1.17×
20.5 (21.0)†	1.60×	1.70×	1.60×	1.81×
23.0.....	1.53×	1.81×	1.48×	1.74×
27.0 (28.0).	1.87×	1.92×	1.78×	1.90×
31.0 (32.0).	1.60×	1.99×	1.93×	2.22×
35.0 (34.0).	1.53×	1.33×	1.65×	1.88×

* Each figure is average of ten sections or of eighty cells.

† Values in parentheses are temperatures during 36-hour test where they differed from those of 24-hour test.

To reduce the possibly complicating effects of cell division in the excised sections, a second group of experiments was completed in periods of 24 and 36 hours (table 3). During these shorter periods no evidence of cell division was obtained, cell elongation being of the same magnitude as section elongation, within the limits of variability of the materials used. In the range from 0° to 20° C., the temperature coefficients (table 4) were again above 2.0 in both experiments, indicating that the limiting process or processes at these temperatures were chemical. The drop in the growth rates at temperatures above 20° C., with less actual growth above 30° to 32° C., is probably related to a low optimum temperature for growth but may be affected also by high respiration and low rates of gas exchange in the partially submerged sections. High temperatures caused injury, as indicated by loss of turgidity and discoloration of scape sections, at temperatures above 32° C.

CASTOR BEAN LEAVES.—It is reasonably certain that cells are no longer dividing in nearly grown leaves of *Ricinus communis* and that expansion of the blade in such leaves occurs through enlargement of cells only. The temperature coefficients of cell enlargement can thus be obtained from the rates of leaf growth at different temperatures. Young plants, which had been grown in large pots in the greenhouse, were placed in constant-temperature incubators in darkness. The data of table 5 show the enlarge-

TABLE 4

TEMPERATURE COEFFICIENTS OF CELL ELONGATION IN SECTIONS OF DANDELION SCAPE IN SUCROSE-IAA SOLUTION (CALCULATED FROM CHANGES IN SECTION LENGTH)

Temperature range (° C.)	Q_{10} (24-hour period)	Q_{10} (36-hour period)
0.5–10.0.....	2.84	2.16
5.0–14.0.....	4.45	2.16
10.0–20.5 (10.0–21.0)....	2.13	2.14
14.0–23.0.....	1.07	0.96
20.5–27.0 (21.0–28.0)....	1.77	1.45
23.0–31.0 (23.0–32.0)....	1.17	2.09
27.0–35.0 (28.0–34.0)....	0.54	0.73

ment of nearly grown leaves at three temperatures. Growth rates were satisfactorily high during short periods in the dark but tapered off sharply when the plants remained without light for longer intervals. The Q_{10} values (table 6) in both temperature ranges were high (2.0+) in all three experiments, except in the 22°–28° C. range of the first experiment, in which moisture may have been limiting (6). In general, these results seem to confirm the findings of THUT and LOOMIS (9) that expansion of such leaves shows high temperature coefficients, suggesting the importance of chemical processes in cell enlargement.

TABLE 5

ENLARGEMENT OF CASTOR BEAN LEAVES AT THREE TEMPERATURES

TEMPERATURE (° C.)	EXPERIMENT I			EXPERIMENT II		EXPERIMENT III (17 HOURS)
	First period (9 hours)	Second period (15 hours)	Third period (11 hours)	First period (14 hours)	Second period (11 hours)	
10	0.21*	0.30	0.05	0.22	0.18	0.17
22	0.90	0.80	0.33	1.90	0.34	0.65 (20° C.)
28	0.96	0.82	0.30	2.72	0.50	2.66 (30° C.)

* Each value is average increase in cm. of lengths of five main veins of a nearly grown leaf on each of three plants.

TABLE 6

TEMPERATURE COEFFICIENTS OF ENLARGEMENT OF CASTOR BEAN LEAVES

TEMPERATURE (° C.)	EXPERIMENT I			EXPERIMENT II		EXPERIMENT III (17 HOURS)
	First period (9 hours)	Second period (15 hours)	Third period (11 hours)	First period (14 hours)	Second period (11 hours)	
10–22 (10–20)	3.35	2.26	4.79	5.11	1.64	3.82
22–28 (20–30)	1.12	1.03	0.85	2.59	2.94	4.09

TABLE 7

ELONGATION OF MARKED SECTIONS OF BEAN HYPOCOTYL AT VARYING TEMPERATURES DURING 24-HOUR PERIOD IN DARK

SECTIONS (FROM TOP)	TEMPERATURE (° C.)						
	5	10	15	20	25	30	35
1 (0–4 mm.)	0.5*	0.3	1.5	3.0	1.5	2.3	2.0
2 (4–8 mm.)	0.0	1.0	1.0	4.5	2.0	2.5	2.5
3 (8–12 mm.)	0.0	0.0	0.5	6.0	2.0	2.0	2.5
4 (12–16 mm.)	0.0	0.0	0.5	7.5	1.8	2.3	8.5
5 (16–20 mm.)	0.0	0.0	1.5	5.8	3.8	4.5	7.8

* Figures are average mm. increases in length of sections originally 4 mm. long. Two seedlings at each temperature except 5° and 15° C. with one seedling each.

BEAN HYPOCOTYLS.—The growth of hypocotyls of *Phaseolus vulgaris* at different temperatures was observed in small seedlings which had been germinated in the dark in sand, marked into uniform regions with India ink, covered with black paper cones to exclude light, and placed at temperatures ranging from 5° C. to 35° C. It was determined that cell division in the hypocotyl was limited to the upper 2–3 mm. (section 1). By calculating the rates of section elongation in regions of the hypocotyl in which cells were no longer dividing (sections 2 through 5), the influence of temperature on cell elongation was measured (table 7). Each section was 4 mm. in length at the beginning of the 24-hour test period. In general, maximum elongation of the sections occurred at 20° C.

In sections 2 through 5, in which growth was assumed, on the evidence from parallel plants, to have wholly resulted from elongation of cells, high temperature coefficients were found for the ranges 10°–20° and 15°–25° C. (table 8). The Q_{10} values for these ranges were all over 2.0. In the range 20°–30° C., the values were less than 1.0, possibly indicating that cell moisture was not maintained in the exposed sections. During a 72-hour growth period, the temperature coefficients of section elongation showed the same trends. Q_{10} values of cell elongation (sections 2–5) were above 2.0 in the temperature ranges 10°–20° C. and 15°–25° C. In the range 20°–30° C., the coefficients on the whole were low (below 1.0).

Some of the irregularity of results may be attributed to the small number of plants used in any one experiment and, for the data shown in tables 7 and 8, to the exceptional growth made by the plants at 20° C. The conclusion from all the experiments, however, is clearly that there is a high temperature coefficient for cell elongation in bean hypocotyl up to temperature values of 30° C.

Discussion

Chemical reactions affecting the rate of cell enlargement might be localized in the cytoplasm, in the cell membrane, or in the wall. One of us has shown (3) that enlargement in the cells of the cotyledon of *Brassica oleracea* var. *italica* may be checked by a deficiency of soil nutrients and be resumed if these elements are again made available. At least an indirect effect of a protoplasmic reaction is indicated. In the experiments with *Taraxacum* scapes reported here, however, rapid cell enlargement and some cell division occurred without the further addition of nitrogen or other soil nutrient elements. Studies with attached dandelion scapes (3) showed that early rapid growth was accompanied by a marked increase of protein nitrogen per cell but that the same fraction decreased on a per cell basis during the second spurt of growth at the time of fruit maturity. It thus seems probable that any relationships between protoplasm synthesis and cell enlargement are indirect rather than direct. The relationships shown by

TABLE 8

TEMPERATURE COEFFICIENTS OF ELONGATION OF MARKED SECTIONS OF BEAN HYPOCOTYL DURING A 24-HOUR PERIOD OF GROWTH IN DARK

TEMPERATURE RANGE (° C.)	SECTION				
	1	2	3	4	5
5–15	3.00	.....	.....	.....	.....
10–20	10.00	4.50	.....	.....	.....
15–25	1.00	2.00	4.00	3.60	2.51
20–30	0.78	0.56	0.33	0.31	0.78
25–35	1.33	1.25	1.25	4.72	2.05

AVERY *et al.* (1, 2) between protein synthesis and hormone synthesis could be a factor in cell enlargement, and it is probable that respiratory energy is either a directly or indirectly contributing factor.

Marked temperature effects on the shrinking and swelling of plant tissue in water have been noted by a number of authors. DELF (4), studying the influence of temperature on the permeability of protoplasm to water, found that the permeability of dandelion scape cells to water, as measured by rate of tissue shrinkage in sucrose solutions, was doubled or tripled with a 10° rise in temperature between 10° and 40° C. STILES and JORGENSEN (8) studied the influence of temperature on the swelling of potato tissue in water and calculated that the Q_{10} values for this reaction ranged from 2.7 to 3.0 between 10° and 30° C. The rate of swelling of carrot tissue in water within the same temperature range showed coefficients of 1.3–1.6. The process of water intake into potato tuber tissue was further investigated by REINDERS (7), who found that absorption of water by aerated slices was stimulated by indoleacetic acid, the reaction having a Q_{10} of 2–3. The effect of temperature on the swelling of mature storage tissue is probably complex, and even DELF's experiments may involve more than simple permeability changes. A possible effect of temperature on permeability would seem, however, to be a factor which should be considered in explaining the effects of temperature on cell enlargement.

Advocates of the theory of growth by intussusception might be of the opinion that a high temperature coefficient is evidence for this mechanism. Any system of adding cellulose or pectic units to an extending cell wall, however, or any growth-hormone action increasing the plasticity of the cell walls (5), would involve enzymatically controlled, chemical reactions. The present evidence does not justify the choice of any one of them.

Whatever reaction or group of reactions may prove to be concerned, the data obtained indicate that chemical reactions are generally limiting for cell-enlargement processes. Such a conclusion does not, of course, eliminate the presence of physical forces, but it does indicate that they are not controlling in the tissues studied.

Summary

The enlargement of nondividing cells of scape sections of *Taraxacum officinale* in a sucrose-hormone solution, of cells of attached, nearly grown leaves of *Ricinus communis*, and of hypocotyls of *Phaseolus vulgaris* seedlings, showed the temperature coefficients of a chemical reaction over temperature ranges between 0° and 30° C. Such Q_{10} values are taken as evidence that chemical reactions in the protoplast and/or the extending wall, rather than the physical processes of osmosis or imbibition, are limiting for cell enlargement under conditions of an adequate moisture supply.

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RELATION OF LIGHT INTENSITY TO EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON WATER HYACINTH AND KIDNEY BEAN PLANTS

WILLIAM T. PENFOUND AND VIRGINIA MINYARD

In one study on the effect of formagenic herbicides on water hyacinth, we utilized the droplet method. The use of droplets of the butyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D) in kerosene proved very advantageous because of the rapid spreading of the droplets, the speedy entrance of the material into the tissues, and the relatively rapid destruction of the organism. Tests with droplets of kerosene alone showed only a very slight necrosis at the site of application.

In one experiment much greater epinasty of leaves and subsequent necrosis developed in the laboratory than in full sunlight. Thinking that light intensity might be a determining factor, we treated sets of plants in the laboratory, under a table out of doors, and on top of a table out of doors. In a somewhat different experiment leaves of parent plants having three offshoots were treated in the laboratory and under field conditions. In both experiments the plants were similar in size and were removed from the same habitat (tubs in full sunlight) just prior to application of the herbicide and transferred to the experimental condition desired. With all treatments the same pipette was used in order to obtain uniform droplet size and the same concentration (1000 p.p.m.) of the herbicide. Each plant was treated with four droplets, one on each of four different leaves, giving a total of 200 μ gm. per plant.

Daily observations were made on the degree of epinasty and on the percentage destruction. The results on water hyacinth (table 1) include the data at the end of the experiments (14 days) only. Greater epinasty and much greater necrosis occurred in shaded conditions, whether small, medium, or large plants were employed. Complete destruction did not occur in any plants in full sunlight, although several leaves of small plants exhibited considerable necrosis. In the shade the parent plant was killed quickly, and all the offshoots showed considerable necrosis by the end of the experimental period. In full sunlight, however, only a few leaves of the parent plant were killed, the offshoots not only being uninjured but increasing in number by 300%.

It is obvious that these observations concerning the apparent influence of light on the responses of water hyacinth

to the butyl ester of 2,4-D in kerosene differ conspicuously from those of MITCHELL and BROWN (1) and WEAVER and DE ROSE (2). MITCHELL and BROWN found that no bending of the stems occurred when leaves of snap bean plants growing in darkness or in shade were treated with aqueous solutions of 2,4-D in Carbowax 1500. WEAVER and DE ROSE found that "most stem curvature occurred in plants growing in the light" when Red Kidney beans were treated with the ammonium salt of 2,4-D. In all our work, however, the degree of epinasty and the destruction of water hyacinth plants were greater in the shaded plants.

Since it was felt that the greater destruction of water hyacinth in shade (versus sunlight) might indicate a specific difference, we tested Red Kidney beans with the same phytocide. Twenty-four plants in the late cotyledon stage were potted, allowed to develop for 3 days, and then divided into two groups, one of which was placed in full sunlight and the other in darkness for 48 hours. Upon testing, a moderate amount of starch was found in the leaves of those exposed to sunlight, but none was found in plants in darkness. Each plant was then treated, under identical conditions, with four drops of the butyl ester of 2,4-D in kerosene at a strength of 1000 p.p.m. The starch-present and starch-free plants were then divided into three groups of four plants each, these groups being disposed on the same table and exposed to light as follows: (*A*) by a window with a southwest exposure; (*B*) in diffuse light; and (*C*) in darkness. The treatments under each light condition were as follows: (*a*) control; and two drops each on (*b*) cotyledons, (*c*) primary leaves, and (*d*) primary leaves of plants from which cotyledons had been removed.

TABLE 1

RELATIVE EFFECT OF BUTYL ESTER OF 2,4-D IN KEROSENE (FOUR DROPS OF 1000 P.P.M. PER PLANT) ON SMALL, MEDIUM, AND LARGE PLANTS OF WATER HYACINTH IN DIFFUSE LIGHT AND IN FULL SUNLIGHT (14 DAYS AFTER TREATMENT)

PLANT	EPINASTY		NECROSIS (%)	
	Shade	Sunlight	Shade	Sunlight
Small (leaves 14 in.)	Marked	Marked	90	30
Medium (leaves 21 in.)	Marked	Moderate	40	5
Large (leaves 44 in.)	Marked	Slight	30	None
Medium plants with offshoots	Marked	Moderate	100 (parent); 30 (offshoot)	20 (parent); 0 (offshoot)

In about 3 hours inrolling was quite evident on all treated leaves. In 6 hours some bending occurred in the first internodes, appearing first on those plants in which the cotyledons were treated. There was also considerable epinasty or hyponasty of the primary leaves, but, since bending was either inward or outward, this phenomenon was not used as a measure of phytocidal activity. Local necrosis of leaves occurred in about 24 hours, but general necrosis did not supervene until the third day. We have utilized the bending of the first internode and the death of plant parts as our major criteria of phytocidal effectiveness.

In all cases the controls showed no ab-

normalities, even in darkness. The reactions of plants on which only the cotyledons were treated were similar to those of leaf-treated plants except that bending occurred slightly earlier and inrolling of the leaves developed slightly later. From our observations it appears that there is no basic difference in phytocidal reaction between cotyledon-treated and leaf-treated plants with the materials and dosages utilized. Removal of the cotyledons apparently had no effect whatever on the reactions of plants to the phytocide. We are, therefore, presenting only the data on the entire plants in which the primary leaves were treated (table 2).

TABLE 2

RELATION OF LIGHT TO PHYTOCIDAL EFFECTIVENESS (ON RED KIDNEY BEAN PLANTS) OF BUTYL ESTER OF 2,4-D IN KEROSENE

Days after treatment	Direct light		Diffuse light		Darkness	
	Bending	Necrosis (%)	Bending	Necrosis (%)	Bending	Necrosis (%)
	In light before treatment (starch in leaves)					
1	10°	None	45°	None	30°	None
3	90	30	80	30	45	35
7	90	90	*	*	80	80
	In darkness 48 hours before treatment (no starch)					
1	5°	None	10°	None	30°	None
3	20	10	180	70	90	30
7	30	70	*	*	*	*

* Dead, prostrate.

Bending occurred in nearly all treated plants by the end of the first day (table 2). With the concentration used, it increased with time and was followed by considerable necrosis by the third day. By the end of the seventh day nearly all the plants were dead, and there was little difference among those in direct light, diffuse light, and darkness except that the starch-free plants in direct light fared somewhat better than the others (table 2). With one exception (starch-present plants in darkness) plants in darkness and diffuse light were killed more readily than those in direct sunlight. This checks closely with our experience with water hyacinth under both laboratory and field conditions.

The reasons for the difference between our results and those of MITCHELL and BROWN (1) and WEAVER and DE ROSE (2) when using the same or very similar species are not entirely clear. We have read their reports carefully and are convinced of the care of their experimentation and of the accuracy of their results. It is possible that the greater amounts of materials used in our experiments (200 μ gm. versus 10–50 μ gm. of herbicide per plant) may account in part for the difference. That the stimulus travels with

soluble carbohydrate is called into question, however, since the greatest bending and the greatest destruction of our Red Kidney bean plants occurred in starch-free plants in diffuse light and in darkness. In one experiment WEAVER and DE ROSE found that one drop of an aqueous solution of the ammonium salt of 2,4-D (1000 p.p.m.) did not induce stem curvature when applied to the cotyledons of the soybean except when they were scratched. This was not true for our Red Kidney bean plants when treated with four drops of the butyl ester (1000 p.p.m.). It is possible that the diversity in results was due to a specific difference, to the greater total amount of 2,4-D used by us, or to the fact that the butyl ester penetrated the cotyledons much more readily than the ammonium salt. In any event, it is certain that the butyl ester of 2,4-D in kerosene is effective on cotyledons and in darkness as well as in sunlight. These facts may be related to the greater penetrating capacity of the carrier (kerosene).

Summary

1. Water hyacinth plants in shade and full sunlight were treated with four droplets each (1000 p.p.m.) of the butyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D) in kerosene.

2. Greater epinasty and much greater necrosis occurred in plants under shaded conditions than in those placed in full sunlight.

3. Red Kidney bean plants were treated with four droplets each (1000 p.p.m.) of the butyl ester of 2,4-D in kerosene, either on the cotyledons or on the primary leaves, and placed in darkness, in diffuse light, or in direct sunlight.

4. Bending and necrosis were similar in cotyledon-treated and leaf-treated plants and in darkness, diffuse light, and direct sunlight except that survival was somewhat better in direct sunlight.

5. It is suggested that the effectiveness of the phytocide used in this experiment may have been enhanced by the penetrating capacity of the carrier (kerosene).

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LITERATURE CITED

1. MITCHELL, J. W., and BROWN, J. W. Movement of 2,4-dichlorophenoxyacetic acid stimulus and its relation to translocation of organic food materials in plants. BOT. GAZ. **107**:393–407. 1946.

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CURRENT LITERATURE

Growth Regulators for Garden, Field, and Orchard. By JOHN W. MITCHELL and PAUL C. MARTH. Chicago: University of Chicago Press, 1947. Pp. 129+16 figs.+5 tables. $2.50.

During the past decade progress in the study of the effects of organic chemicals, often referred to as synthetic plant hormones or growth-regulating substances, has been phenomenally rapid. There is scarcely a phase of plant production in which the use of growth-regulating substances has not already become of significance. The field of their use is already a very broad one and is rapidly expanding. The future use and application of growth-regulators may eventually extend into every phase of plant production, storage, and utilization where living plants are concerned.

Up to the present time information concerning them has been available only through technical papers or a few popular articles widely scattered in publications dealing with plant culture. The present book is most timely in that it has been written by two of the most active contributors on both the theoretical and the applied aspects of the use of growth-regulators. It furnishes in the applied field an authentic summary of the information available up to date in clear, direct style and language. Chapters are devoted to weed control, vegetative propagation and transplanting, the preventing of growth in stored plant material, the prevention of premature dropping of fruits, the improvement of fruit-set and the production of seedless fruit, and a composite chapter dealing with other plant responses to these compounds. There is a concise and comprehensive index.—E. J. KRAUS.

Heredity and Variation in Microorganisms. ("Cold Spring Harbor Symposia on Quantitative Biology," Vol. XI.) Cold Spring Harbor, N.Y.: Biological Laboratory, 1946. Pp. 314. $6.00 (plus postage).

The wide scope of this series of papers and the intensive treatment afforded each of the many aspects of heredity and variation in micro-organisms impose on the reviewer an exceedingly difficult task of summation and abstraction; on the other hand, the inclusion in the volume of the comments and criticisms by those attending the symposium and best qualified to criticize relieves him in large part of his other important function, that of evaluation.

The Cold Spring Harbor Symposium of 1946 comprises, as usual, a comprehensive and up-to-the-minute review of the designated field of the biological sciences by its outstanding experimentalists. The individual papers are short and concise, and for the most part the material is exceedingly well presented.

Although there is a great diversity of experimental work included in this volume, the greater number of papers deal with spontaneous and induced mutations in fungi, bacteria, and viruses and with the use of such mutant strains in the elucidation of biochemical processes, pathogenic manifestations, etc., in the living cell (or unit). Recent developments in the study of genic control of biochemical processes in *Neurospora* are reported by W. D. BONNER and FRANCIS J. RYAN. Of particular interest is the rapidly accumulating mass of information on quite comparable phenomena in bacteria and viruses which would appear to be due to genic control. Papers by E. L. TATUM, JOSHUA LEDERBERG and E. L. TATUM, RENÉ J. DUBOS, MARY I. BUNTING, M. DEMEREC and R. LATARJET, S. E. LURIA, and ANDRÉ LWOFF emphasize the similarity, if not identity, of fundamental and intermediate control of synthetic processes, metabolic degradations, etc., in bacteria and in the higher fungi, where, because of the operation of a sexual mechanism, these activities can be shown to be under genic control. Viral mutations, originating spontaneously or as a result of irradiation, affecting various characteristic activities of viruses such as pathogenicity, lysis ability, plaque form, etc., and indicating a similar apparent controlling mechanism, are described in papers by THOMAS F. ANDERSON, M. DELBRUCK and W. T. BAILEY, JR., A. D. HERSHEY, and N. W. PIRIE.

Three of the papers dealing with mutations in bacteria and viruses—those by HERSHEY, LEDERBERG and TATUM, and LURIA—present evidence that in these lowest organisms there exists a mechanism which accomplishes recombination and segregation of genetic factors, the results of which are similar to those of the Mendelian process in higher forms. Along quite different lines a paper by G. PONTECOVO deals with the applicability and the necessary modifications of classical genetical analysis and methods in the study of heterocaryotic systems in the higher fungi and particularly in the Fungi Imperfecti—i.e., in all forms lacking sexual reproduction but commonly showing hyphal fusions and intimate nuclear associations. Thus it would appear that the last frontiers are falling before the expanding utility of genetical techniques in the elucidation of fundamental processes.

The mechanism by which the gene controls the elaboration, duplication, and activity of complex cytoplasmic constituents is the essential feature of papers by S. SPIEGELMAN and CARL C. and GERTRUDE LINDEGREN. In both of these papers the be-

havior of certain self-duplicating yeast characters, which at meiosis do not follow random segregation, in respect to genic constitution and environmental factors constitutes the bases for hypotheses concerning the intermediate steps of genic action on the ultimate activities of the cell. A paper by T. M. SONNEBORN describes the experimental means by which the quantity of a similar (?) self-duplicating cytoplasmic factor (kappa of the gene K-kappa-killer substance complex) can be altered and also the significance of the gene-cytoplasmic factor relationship. The production of distinctive constituents by certain tumor cells is described by JOHN G. KIDD, and the gene-plastid relationship and the evidence for plastid mutations in higher plants are discussed by M. M. RHOADES.

The symposium also includes a number of papers of particular interest dealing with the general subject of heredity and variation in micro-organisms which pertain to matters other than genic mutations and gene-cytoplasmic constituent relationships. Among these are discussions of the following subjects: the need for, and difficulties attending the development of, a natural system of classification of the bacteria, by C. B. VAN NIEL; variations and inheritance in rust fungi, by T. JOHNSON; and complex reproductive processes in bacteria under certain conditions of admixture, by L. DIENES.

A most welcome addition to the symposium, while not contributing directly to the chosen topic of discussion, is a very understandable description of the theory and practice of phase microscopy.

While this volume will be read, in its entirety or in part, with much interest by biologists generally, it must surely be considered required reading for the students of two large fields of biological endeavor: genetics and microbiology.—JOHN R. RAPER.

An Introduction to Plant Anatomy. By ARTHUR J. EAMES and LAURENCE H. MACDANIELS.. 2d ed. New York: McGraw-Hill Book Co., 1947. Pp. xvii+427. Illus. $4.50.

The viewpoint and aims have not changed in this new edition of a standard text. The gross organization of the book has remained the same as in the first edition except that the chapter on the history of plant anatomy has been removed to allow for inclusion of additional descriptive material in the other chapters. Reorganization of the individual chapters varies from little, where essentially no new knowledge has recently come to light, to considerable in those chapters dealing with subject matter which has received much additional study during the last twenty years.

Chapters which have undergone most extensive "modernization" are those on the cell, meristems, tissues and tissue systems, periderm and abscission, leaf and flower, fruit and seed.

In the chapter on the cell a brief discussion of intrusive and symplastic growth is found in addition to that on gliding growth. The material on wall structure has been greatly enlarged in the light of extensive research during the last two decades.

The classification of meristems has been revised, and a discussion of the three main theories of structural development and differentiation—apical cell, histogen, and tunica-corpus—included. Descriptions of stem (vegetative), root, and floral apices, pointing out similarities and differences among them, should be helpful to the student.

The discussion of phloem anatomy has been brought up to date, taking into account the greatly increased knowledge in this area.

Considerably more is now known about the details of abscission—the formation, or lack of formation, of separation layers, periderms, etc.—than was known at the time of the first edition; this information is included particularly in the relatively detailed discussion of specific forms: *Castanea*, *Catalpa*, *Betula*, and *Populus*.

Revision in the chapter on the leaf deals particularly with leaf ontogeny. Discussions of the development of specific leaf tissues from the various meristematic regions and the duration of activity of such meristematic cells are useful additions. In this chapter a new three-dimensional cellular drawing of a typical leaf has been added and should prove a great help to both teacher and student.

Discussion of the vascular anatomy of the flower has been greatly enlarged and brought up to date. It is as comprehensive as such a discussion could be in an elementary text and should even be helpful as a starting-point for more advanced work. The reviewer personally regrets the use of the terms "fusion," "union," "cohesion," and "adnation" throughout this section to refer not only to cases in which actual fusion of initially independent parts does occur in ontogeny but also to those cases in which primordia are never independent and are completely nondiverged from their initiation.

The illustrative material has been increased, particularly in those areas showing the greatest revision. Many of the new figures are excellent photomicrographs used in conjunction with old and new line drawings.

The list of references at the end of each chapter has been brought up to date and considerably enlarged, again in those areas in which most revision has been done.

No attempt has been made in this review to report on the book as a whole, since it is assumed that those interested are familiar with the great usefulness of the original edition and are acquainted with the authors' clear presentation. A summary of changes made in this new edition should therefore merely point out the increased value of this already favorite text.—BARBARA F. PALSER.